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14. ABSTRACT The goal of this project is to investigate the role of long chain fatty acyl-CoA synthetase 4 (ACSL4) as a biomarker for, and mediator of an aggressive breast cancer phenotype. Results indicate that expression of ACSL4 in breast cell lines and tumor samples is inversely correlated with expression of estrogen receptor, progesterone receptor, androgen receptor and human epidermal growth factor receptor 2 (HER2). Breast cancer lacking all 4 receptors (quadruple negative breast cancer (QNBC)) can be identified by ACSL4 expression status with a sensitivity of 78% and a specificity of 86%. In addition, in vitro data from experiments in breast cancer cell lines demonstrates that simultaneous expression of a receptor and ACSL4 is characterized by resistance to treatment with a receptor-based targeted therapy, such as tamoxifen or lapatinib. Forced expression of ACSL4 results in increased proliferation, migration and invasion. The effects are specific for ACSL4 in that neither a mutant ACSL4 lacking enzyme activity nor ACSL3 are effective. Messenger RNA and protein expression data indicate possible effectors of the action of ACSL4.					
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FINAL REPORT

1. INTRODUCTION

The subject of this project is the role of the fatty acid activating enzyme, long-chain acyl-CoA synthetase 4 (ACSL4), in contributing to the malignant phenotype in human breast cancer. Fatty acid activation involves the condensation of a CoA molecule with a fatty acid, and is a requirement for subsequent incorporation of the fatty acid into triglycerides and phospholipids as well as for oxidation of fatty acids to provide energy. ACSL4 is one of 5 mammalian isoforms of the enzyme and is characterized by specificity for arachidonic acid as a substrate. Data derived from mining public information indicates that ACSL4 is differentially expressed in estrogen receptor alpha-negative (ER-) breast cancer. Using this information as a starting point, we have investigated several different aspects of the relationship between ACSL4 expression and various characteristics of breast cancer, both *in vitro* and *in vivo*.

The research supported by this award for the period August 15, 2013 through August 14, 2015 has resulted in the generation of the data described below. The published data can be found in references [1] and [2], which are attached in Appendix A. It is also summarized briefly below. The "Statement of Work" outlined in the proposal described 5 tasks associated with 3 specific aims. Progress with respect to each of these tasks is detailed below. Those studies carried out during the past year appear at the end of each section.

2. KEYWORDS

Fatty acid metabolism; triple negative breast cancer (TNBC), Receptor-positive breast cancer (RPBC), long chain fatty acyl-CoA synthetase (ACSL); perilipin (PLIN); estrogen receptor (ER); androgen receptor (AR); molecular subtype

3. OVERALL PROJECT SUMMARY

Specific Aim #1: Determination of the specific biochemical function of increased ACSL4 activity in ER- breast cancer cells in tissue culture

Task 1: Develop an MCF-7 cell line with inducible ACSL4 expression

This task has been completed and expanded to include ER-negative, HER2-positive SKBr3 cells as well, as detailed in reference [2]. A combination of *in vitro* and *in vivo* experiments were carried out to assess the effects of ACSL4 expression on proliferation (figures 2, 6 and 7), anchorage-independent growth (figure 3 in reference [2]) and invasion (figure 4 in reference [2]). Sensitivity to hormone-based targeted treatments was also assessed figure 8 in reference [2]). Results are consistent with ACSL4 expression contributing to the manifestation of an aggressive breast cancer phenotype. In addition, a combination of mRNA expression microarray studies (tables 6 through 8 in reference [2]) and proteomic pathway array analysis (table 9 in reference [2]) lead to the observation that the autism susceptibility candidate gene 2 (AUTS2) and inhibition of beta catenin degradation may play a role in mediating the effects of ACSL4 (figure 9 in reference [2]).

During the past year we have continued an *in silico* analysis of public databases, including Oncomine [3] and NCBI's Gene Expression Omnibus in order to generalize the fatty acid metabolic landscape in breast cancer beyond ACSL4. Table 1 details the expression pattern of mRNA for additional proteins involved in fatty acid metabolism with respect to relative expression in receptor-positive (RPBC) versus triple negative breast cancer (TNBC) specimens and cell lines. Five representative studies analyzing tissue specimens (Chin [4], Curtis [5], Hatzis [6], Richardson [7] and

TCGA [8]) and one analyzing breast cancer cell lines [9] are included. The proteins highlighted in red are predominant in TNBC, while those in black are more highly expressed in RPBC.

Table 1: Fatty Acid Metabolism in Triple-negative Compared with Receptor-positive Breast Cancer

Protein	STUDY											
	Chin		Curtis		Hatzis		Richardson2		TCGA		Neve	
	FC	pV	FC	pV	FC	pV	FC	pV	FC	pV	FC	pV
ACLY	-1.25	0.013	-1.22	2.56e-14	-1.08	0.008	-1.53	0.007	-1.13	0.019	-1.16	0.092
ACACA	-1.22	1.30e-05	-1.21	1.81e-09	-1.08	1.12e-04	-1.49	0.038	-1.25	0.003	-1.14	0.014
FASN	-1.86	0.003	-1.60	7.83e-16	-1.72	2.33e-15	-6.37	3.12e-08	-2.22	1.20e-14	-2.11	1.36e-04
SCD1	-1.65	0.006	-1.27	1.15e-06	-1.52	6.25e-08	-2.17	0.004	-2.23	1.07e-07	-1.82	0.002
ACSL1	1.15	0.184	1.29	3.02e-09	1.29	8.01e-06	1.67	0.071	1.27	0.017	-1.33	0.087
ACSL3	-1.69	6.22e-04	-1.16	2.21e-07	-1.38	1.25e-07	-1.43	0.044	-1.39	6.75e-04	-1.69	0.007
ACSL4	1.40	8.46e-04	1.43	1.13e-28	1.13	5.93e-10	1.84	0.002	1.64	8.40e-09	1.92	1.90e-05
ACSL5	1.34	0.002	1.26	3.65e-09	1.07	0.002	1.44	0.122	1.61	1.12e-04	1.05	0.321
ACSL6	1.45	0.010	1.07	1.29e-08	1.05	0.002	1.92	0.004	2.0	8.14e-04	-1.01	0.413
PLIN2	1.68	2.22e-05	1.94	6.02e-36	1.88	6.81e-21	1.70	0.016	2.17	8.13e-14	1.62	0.023
LPIN1	2.43	4.76e-08	2.19	8.21e-55	1.85	6.62e-27	3.16	2.64e-06	2.98	1.40e-15	1.34	0.014
DGAT2	NA	NA	1.33	1.14e-24	NA	NA	7.49	5.47e-09	3.23	1.77e-14	NA	NA
PLA2G4A	3.13	9.26e-05	1.07	6.31e-09	1.33	4.11e-09	6.47	1.69e-04	3.91	3.07e-12	1.38	0.05
CPT1A	-1.65	4.08e-05	-1.07	4.78e-12	-1.20	5.80e-09	-2.21	0.001	-1.72	4.05e-10	-2.46	6.51e-07
ACOX2	-4.11	1.36e-18	-3.97	8.20e-74	-1.62	1.70e-23	-7.37	1.89e-04	-5.87	5.82e-23	-1.61	0.009
AMACR	-1.26	4.45e-05	-1.21	7.89e-23	-1.09	1.46e-07	-1.97	1.54e-04	-1.78	1.49e-09	-1.08	0.055
GPAM	NA	NA	1.09	0.293	NA	NA	1.09	0.335	1.13	0.040	NA	NA
CHKA	1.08	0.269	1.07	0.003	1.11	9.64e-04	1.11	0.310	1.52	1.40e-05	1.02	0.450
FABP5	2.29	3.05e-06	2.47	5.66e-44	2.60	1.15e-23	4.38	1.48e-05	3.58	3.59e-21	5.41	0.007
FABP7	8.27	2.86e-04	2.80	0.013	2.37	7.49e-14	78.03	1.41e-07	23.50	6.59e-16	-1.39	0.08

FC = Fold change in triple negative samples versus receptor-positive samples.

pV = p Value

A distinct pattern can be observed. RPBC appear to rely on *de novo* fatty acid synthesis (ACLY, ACACA, FASN and SCD1) and fatty acid oxidation (ACSL3, CPT1A, ACOX2 and AMACR), while TNBC upregulate the ability to acquire (FABP5 and FABP7), store (PLIN2, ACSL4, LPIN1 and DGAT2) and subsequently reutilize (PLA2G4A) exogenous fatty acids. These data provide a snapshot of potential differences in fatty acid metabolism between RPBC and TNBC.

In order to further validate these differences, we analyzed the effect of silencing expression of the estrogen receptor (ER α) on the pattern of expression of proteins

Table 2: Effect of ER α Silencing on Expression of Fatty Acid Metabolic Genes

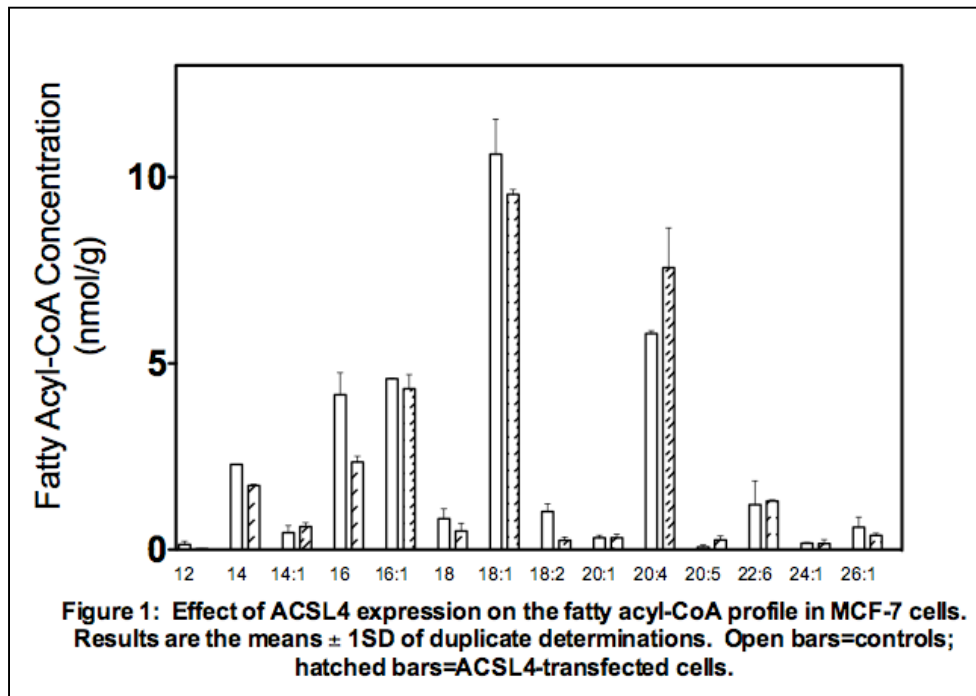
Gene	Control	Silenced	Fold Change	pValue
ACLY	1201 \pm 117	1896 \pm 146	1.66	0.003
ACACA	387 \pm 17	95 \pm 2	-4.00	9.08e-06
FASN	2863 \pm 353	942 \pm 327	-3.03	0.002
SCD1	7181 \pm 86	3606 \pm 281	-2.00	3.02e-05
ACSL1	1517 \pm 60	631 \pm 43	-2.43	3.21e-05
ACSL3	2043 \pm 41	1684 \pm 96	-1.21	0.004
ACSL4	150 \pm 5	1867 \pm 43	12.44	2.30e-06
ACSL5	24 \pm 0.5	933 \pm 36	38.87	1.81e-06
ACSL6	14 \pm 1	12 \pm 0.5	-1.17	0.101
PLIN2	95 \pm 9	575 \pm 86	6.04	6.70e-04
LPIN1	86 \pm 6	69 \pm 22	-1.25	0.266
DGAT2	187 \pm 18	154 \pm 10	-1.21	0.049
PLA2G4a	9 \pm 0.5	127 \pm 7	14.11	1.17e-05
CPT1A	141 \pm 7	23 \pm 2	-6.25	1.06e-05
ACOX2	96 \pm 7	759 \pm 61	7.90	5.03e-05
AMACR	135 \pm 10	59 \pm 4	-2.27	2.70e-04
GPAM	132 \pm 26	516 \pm 169	3.90	0.017
CHKA	118 \pm 12	544 \pm 35	4.61	4.02e-05
FABP5	1566 \pm 54	4101 \pm 269	2.61	8.94e-05
FABP7	13 \pm 0.7	11 \pm 0.100	-1.19	0.005

Data shown are mRNA expression values in arbitrary units.

involved in fatty acid metabolism. Table 2 lists the results. Data were taken from a study by Al Saleh *et al* [10]. The genes highlighted in red increased expression of mRNA as a result of ER α silencing. Again we see changes in expression of fatty acid metabolic genes that are consistent with transition to a more aggressive subtype, i.e, from RPBC to TNBC.

Task 2: Assessment of lipid metabolism in control- and ACSL4-induced MCF-7 cells

In collaboration with William Blaner from Columbia University we have determined that expression of ACSL4 results in an increase in arachidonoyl-CoA and a decrease in palmitoyl-CoA levels, a finding consistent with the known enzymatic activity of ACSL4 (Fig 1).



In addition, during the past year we have assessed the effect of acute induction of ACSL4 in MCF-7 cells with respect to both CPT1A and PLIN2 expression. CPT1A activity is the rate-limiting step in mitochondrial β -oxidation of fatty acids, while PLIN2 expression is positively associated with the presence of lipid droplets and functions as a gate keeper for acyl-glyceride lipases. When ACSL4-negative MCF-7 cells are forced to express ACSL4, CPT1A mRNA expression is decreased by 60% ($p=9.4e-04$) and PLIN2 mRNA expression is increased by 102% ($p=9.4e-03$) (unpublished data). Given that forced expression of ACSL4 results in MCF-7 cells displaying a more aggressive phenotype, reducing expression of ER and PR and increasing resistance to apoptosis, it is not surprising that an enzyme associated with RP cancers, such as CPT1A, decreases expression, while one associated with TNBC, such as PLIN2, increases expression.

Task 3: Determine the effect of knocking down ACSL4 in MDA-MB-231 on lipid metabolism

Using siRNA specific for ACSL4, we were able to reduce expression of ACSL4 protein by >95% as shown in our publications (figure 5 in reference [1], and figure 4 in reference [2]). Under these conditions, there were no changes noted in either triglyceride or phospholipid metabolism, and no changes in fatty acid oxidation, even using arachidonic acid as substrate (data not shown). We have not yet determined whether the fatty acid profile of the triglycerides and phospholipids is altered, just as we have not yet measured the identities of the specific acyl-CoA's in knocked-down MDA-MB-231 cells. However, these data have been generated for the knock-in models described above.

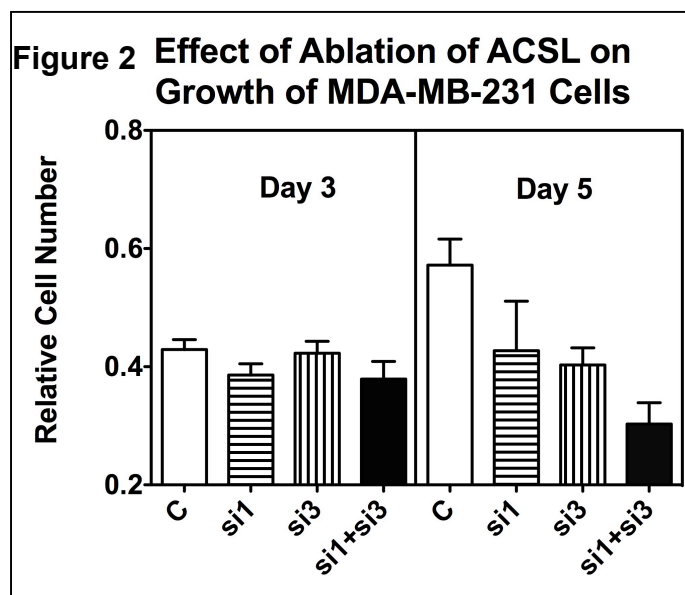
Specific Aim 2: Determination of the potential of ACSL4 as a target for development of chemotherapeutic regimens

Task 4: Assessment of the role of ACSL4 in mediating cell proliferation, migration and invasion.

These experiments essentially revolve around determining the effect of targeting ACSL4 in breast cancer cells. Two models are utilized: 1] the ACSL4+ cell line, MBA-MB-231, and 2] the ACSL4- cell line, MCF-7. We initially compared cell proliferation, migration and invasion in control and ACSL4 knock-down (KD) MDA-MB-231 cells. As previously demonstrated (figure 5 in reference [1]), treatment of MDA-MB-231 cells with ACSL4-specific siRNA results in a >95% decrease in expression of ACSL4 protein. This alteration has no effect on the growth rate of the cells, as shown in the same figure. However, treatment of cells with a pharmacologic inhibitor of ACSL4, triacsin C, which also inhibits ACSL1 and ACSL3, blocked proliferation of the cells. Furthermore, ablation of ACSL4 rendered the cells more sensitive to triacsin C (figure 5 in reference [1]). In addition, as described in figure 4 in reference [2], cell invasion is negatively impacted by a reduction in ACSL4 protein.

Alternatively, we measured the effect of induction of ACSL4 expression in MCF-7 cells, and determined that in this situation, ACSL4 appeared to positively impact the growth rate, as documented in figure 2 in reference [2]. Taken together, these data suggest that cells normally expressing ACSL4 have additional alterations, such as increased activity in alternate growth pathways that render the effect of ACSL4 on growth imperceptible. However, cells, such as MCF-7, which are induced to express ACSL4 in isolation, manifest an effect of this expression on growth. This interpretation is supported by our published observation that a boost in the activity of the MAPK pathway in MCF-7 cells caused by overexpression of cRAF results in induction of ACSL4 expression (figure 4 in reference [1]).

Since it is clear that ACSL4 activity is not required for growth of breast cancer cells, although does increase proliferation under certain circumstances, we evaluated the role of ACSL1 and ACSL3 in supporting proliferation. Results from preliminary studies suggest that these two isoforms play a larger role in sustaining growth, as demonstrated in Figure 2. Ablation of ACSL1 and ACSL3 expression inhibited growth of MDA-MB-231 cells, and the effects were additive. Similar results were seen in MCF-7 and MDA-MB-453 cells (data not shown). Thus, while targeting ACSL4 might be expected to interfere with the metastatic potential of the aggressive QNBC that express it, targeting other isoforms might be effective in limiting the growth of all breast cancer subtypes.



Specific Aim #3: Determination of the prognostic potential of ACSL4 expression

Task 5: Assessment of ACSL4 protein expression in tissue microarrays of breast tumor samples.

An analysis of expression data for both breast cancer cell lines and tumor samples supports the idea that ACSL4 is a biomarker for a subset of aggressive breast cancers, the majority of which are quadruple negative for receptor expression (QNBC). QNBC is that which is triple negative (ER-, PR- and HER-2-negative) as well as AR-negative. Our recent publication illustrates the correlation between biomarker status and ACSL4 expression in both cell lines and tumor samples. An analysis of ACSL4 expression in 71 different breast cancer cell lines indicates that ACSL4 status predicts QNBC with a sensitivity of 78% and a specificity of 86% (tables 1 through 4 in reference [2]). When ACSL4 status is correlated with intrinsic molecular subtype, 100% of claudin-low cell lines are positive, 8% of luminal cell lines are positive and 71% of basal-like cell lines are positive for ACSL4 expression (figure 1 in reference . Based on these findings, we suggest that QNBC breast cancers might be further stratified on the basis of ACSL4 status as a possible prognostic indicator of response to chemo and/or targeted therapies, and have developed preliminary data within the last year to support this hypothesis. For example, the sensitivity of breast cancer cell lines to treatment with the PI3K inhibitor, GSK1059615, is inversely correlated with ACSL4 expression status ($p=0.003$). The opposite is true for treatment with dasatinib ($p=0.01$) (data from Oncomine [3]).

During the past year, using public mRNA expression databases [11], we assessed the value of ACSL4 expression as a prognostic and predictive biomarker. Table 3A indicates that overall, ACSL4 expression is associated with a worse prognosis; however, predicts a better outcome in the more aggressive basal-like subset of breast cancers. Table 2B indicates that this is associated with a better response to chemotherapy, probably due to increased proliferation in ACSL4-positive cancers.

Table 3: ACSL4 as a Biomarker for DMFS at 5 years

A

Molecular Subtype	HR	P value	n
Luminal A	1.56	0.025	235
Luminal B	1.31	0.230	144
HER2	2.46	0.063	62
Basal-like	0.48	0.007	123
All	1.30	0.054	564

B

Therapy	HR	P value	n
Chemotherapy	0.42	0.043	47
Endocrine	1.49	0.155	177
All	1.30	0.054	564

Given that expression of ACSL4 is positively correlated with that of PLIN2, both being overexpressed in TNBC, we also analyzed the prognostic potential of PLIN2 expression. Table 4 indicates that combining PLIN2 mRNA expression with the inverse of PLIN1 is highly predictive of clinical outcome, whether utilizing disease free survival (DFS), distant disease free survival (DDFS) or overall survival (OS) as an endpoint.

Table 4: Predictive value of PLIN2/inversePLIN1 in breast cancer

Parameter	Hazard Ratio	p-value	Patient Cohort	n
DFS	1.46	1.70e-07	All	2652
	1.63	6.73e-05	ER+	1589
	1.28	0.253	ER+/PR+	553
	3.00	1.38e-04	ER+/PR-	132
	0.94	0.660	ER-	517
DDFS	1.90	3.78e-11	All	2007
	2.31	2.99e-08	ER+	1046
	1.58	5.00e-02	ER+/PR+	595
	3.89	4.59e-07	ER+/PR-	180
	1.07	0.695	ER-	4160
OS	1.98	6.16e-13	All	2091
	2.68	9.54e-10	ER+	934
	2.02	4.25e-03	ER+/PR+	438
	2.79	2.41e-03	ER+/PR-	82
	0.98	0.954	ER-	322

The above data suggests that this biomarker endpoint may be especially useful in identifying relative risk among luminal B molecular subtype tumors. Table 5 summarizes survival data when tumor tissue is classified according to intrinsic molecular subtype (PAM-50 [12]).

Table 5: Prognostic value of PLIN2/iPLIN1 as a function of molecular subtype

Classification System	Survival Parameter	Molecular Subtype	HR	P value	n
PAM50*	DFS	Luminal A	0.85	0.540	807
		Luminal B	1.45	1.65e-03	987
		HER2	1.29	0.177	275
		Basal-like	1.04	0.752	419
	DDFS	Luminal A	1.34	0.448	690
		Luminal B	2.16	1.47e-06	657
		HER2	1.15	0.567	180
		Basal-like	1.05	0.753	365
	OS	Luminal A	1.81	0.084	800
		Luminal B	1.78	5.08e-04	678
		HER2	1.61	0.023	237
		Basal-like	1.12	0.564	293

Overall the data we have generated suggest that fatty acid storage, mobilization and metabolism play an important role not only in maintaining the malignant phenotype, but also in establishment of a more aggressive form of breast cancer. Expression of ACSL4 is unique to the more aggressive subtypes, with little or no expression in the receptor positive cancers. PLIN2, while also more highly expressed in the more aggressive phenotypes, is however, differentially expressed in the luminal subtypes and appears to predict risk in luminal B cancers.

4. KEY RESEARCH ACCOMPLISHMENTS

- During the award period we have consolidated our findings concerning the expression of ACSL4 in breast cancer and its relationship to other biomarker status as well as intrinsic molecular subtype. These data, along with the results of forced expression of ACSL4 in a variety of breast cancer cell lines, have been published [2].
- An *in silico* analysis of fatty acid metabolic enzyme expression comparing normal and cancerous tissue as well as intrinsic molecular subtypes indicates that certain patterns of fatty acid enzyme expression are characteristic of breast cancer subtype.
- An analysis of PLIN2 expression in breast cancer and a determination of the prognostic value of PLIN1 and PLIN2 as biomarkers indicate that measurement of these lipid droplet proteins might comprise a powerful biomarker for risk in the ER+ subset of cancers, particularly the luminal B subset.

5. CONCLUSION

As a result of support provided by this award, we have been able to investigate the relationship between ACSL4 expression and breast cancer phenotype, as well as to begin to address the question of the potential utility of ACSL4 as a biomarker for predicting therapeutic response, as well as a target for therapy. We have determined that there is a close relationship between ACSL4 expression and intrinsic molecular subtype, with highest expression in the claudin-low subtype. The absence of expression in a subset of QNBC suggests that ACSL4 status might be useful in further stratifying these samples for purposes of prognosis and treatment. We have also begun investigating the mRNA expression of additional fatty acid metabolic proteins, including the lipid droplet proteins, PLIN1 and PLIN2, in breast cancer samples and cell lines. All in all, we have started to characterize fatty acid metabolism in breast cancer and to determine characteristics unique to receptor expression and molecular subtype. The results of these studies could prove useful in the development of new treatments based on manipulation of fatty acid metabolic pathways.

6. PUBLICATIONS

Peer-reviewed scientific journal:

Monaco ME, Creighton CJ, Lee P, Zou X, Topham MK, Stafforini DM: Expression of Long-chain Fatty Acyl-CoA Synthetase 4 in Breast and Prostate Cancers Is Associated with Sex Steroid Hormone Receptor Negativity. ***Transl Oncol*** 2010, 3(2):91-98.

Wu X, Li Y, Wang J, Wen X, Marcus MT, Daniels D, Zhang DY, Fei Y, Wang LH, Du, Xinxin, Adams S, Singh B, Zavadil J, Lee P, and **Monaco ME**: Long chain fatty acyl-CoA synthetase 4 Is a biomarker for and mediator of hormone resistance in human breast cancer. ***PLoS One*** 2013 Oct 14;8(10):e77060. doi: 10.1371/journal.pone.0077060. eCollection 2013. (PMID: 24155918).

Wu X, Deng F, Li Y, Daniels G, Du X, Ren Q, Wang J, Wang LH, Yang Y, Zhang V, Zhang D, Ye F, Melamed J, **Monaco ME** and Lee P: ACSL4 promotes prostate cancer growth, invasion and hormonal resistance. ***Oncotarget*** 2015, 6 (42): 44849-44863.

Invited Article:

Wu X, Daniels G, Lee P and **Monaco ME**: Lipid metabolism in prostate cancer. *Am J Clin Exp Urol* 2014;2(2):111-120.

7. INVENTIONS, PATENTS AND LICENSES

Title: Methods and compositions for determining the responsiveness of cancer therapeutics

Publication no: US 20120252748 A1

Application no: US 13/432,651

Filing date: March 28, 2012

Inventor: Marie Monaco

Patent Application REJECTED

8. REPORTABLE OUTCOMES

- Verification of the role of ACSL4 as a biomarker for and mediator of an aggressive breast cancer phenotype.
- Elucidation of the association of ACSL4 expression with intrinsic molecular subtype.
- Kaplan-Meier survival analysis to assess the value of lipid enzyme biomarkers.

9. OTHER ACHIEVEMENTS

- Development of several stably transfected breast cancer and prostate cancer cell lines expressing ACSL4
- Applications to NIH and the DoD for funds to continue this research
- Expansion of this project in collaboration with Peng Lee, MD to include an analysis of ACSL4 in prostate cancer (Oncotarget: see above).

10. REFERENCES

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11. APPENDICES:

A: PUBLICATION (NUMBER 1 OF REFERENCES)

B: PUBLICATION (NUMBER 2 OF REFERENCES)

Expression of Long-chain Fatty Acyl-CoA Synthetase 4 in Breast and Prostate Cancers Is Associated with Sex Steroid Hormone Receptor Negativity¹

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Abstract

Previous studies have shown that key enzymes involved in lipid metabolic pathways are differentially expressed in normal compared with tumor tissues. However, the precise role played by dysregulated expression of lipid metabolic enzymes and altered lipid homeostasis in carcinogenesis remains to be established. Fatty acid synthase is overexpressed in a variety of cancers, including breast and prostate. The purpose of the present study was to examine the expression patterns of additional lipid metabolic enzymes in human breast and prostate cancers. This was accomplished by analysis of published expression databases, with confirmation by immunoblot assays. Our results indicate that the fatty acid-activating enzyme, long-chain fatty acyl-CoA synthetase 4 (ACSL4), is differentially expressed in human breast cancer as a function of estrogen receptor alpha (ER) status. In 10 separate studies, ACSL4 messenger RNA (mRNA) was overexpressed in ER-negative breast tumors. Of 50 breast cancer cell lines examined, 17 (89%) of 19 ER-positive lines were negative for ACSL4 mRNA expression and 20 (65%) of 31 ER-negative lines expressed ACSL4 mRNA. The inverse relationship between ER expression and ACSL4 expression was also observed for androgen receptor status in both breast and prostate cancers. Furthermore, loss of steroid hormone sensitivity, such as that observed in Raf1-transfected MCF-7 cells and LNCaP-AI cells, was associated with induction of ACSL4 expression. Ablation of ACSL4 expression in MDA-MB-231 breast cancer cells had no effect on cell proliferation; however, sensitivity to the cytotoxic effects of triacsin C was increased three-fold in the cells lacking ACSL4.

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Introduction

A role for altered lipid metabolism in the genesis of the malignant phenotype is suggested by the increased expression of the fatty acid biosynthetic enzymes fatty acid synthase (FASN) and acetyl Co-A carboxylase (ACC) in a variety of tumors, including those that develop in breast and prostate tissues [1,2]. Whereas FASN and ACC are responsible for *de novo* synthesis of free fatty acids, use of these lipids in subsequent metabolic events, such as glycerolipid synthesis and β -oxidation, requires activation through condensation with a molecule of CoA. There is evidence that activated fatty acids, themselves, can function as transcription factors [3]. The enzymes responsible for the

activation reaction comprise a family of proteins known as fatty acyl-CoA synthetases that are classified according to the chain length of their preferred substrates (short, medium, long, and very long). There are

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five different mammalian isoforms of the long-chain family (ACSL1, 3, 4, 5, and 6), which differ in subcellular localization and substrate specificity [4]. It has been suggested that individual isoforms may serve to channel fatty acids to specific metabolic pathways. ACSL4, for example, is localized to both peroxisomes and mitochondria as a peripheral, rather than integral, membrane protein and has a marked preference for arachidonic and eicosapentaenoic acid as substrates. ACSL4 messenger RNA (mRNA) is highly expressed in placenta, brain, testis, ovary, spleen, and adrenal gland. Relatively low expression levels have been reported in the gastrointestinal tract, including liver, colon, and small intestine [5]. Interestingly, ACSL4 is overexpressed in colon and liver cancer specimens [6,7]. Here, we report that this enzyme is overexpressed in estrogen receptor (ER)–negative, androgen receptor (AR)–negative breast tumors and cell lines, and in AR–negative prostate tumors and cell lines. In addition, our studies suggest that expression of ACSL4 is indicative of steroid hormone-independent growth.

Materials and Methods

Cell Lines and Cell Culture

MCF-7, MDA-MB-231, and MDA-MB-415 cells were obtained from American Type Culture Collection (Manassas, VA). T47D cells were a gift from David Kleinberg of this institution; SKBR3 and BT-20 cells were a gift from Herbert Samuels of this institution; and DU145, PC3, LNCaP, and LNCaP-AI cells were previously described [8,9]. Cells were routinely grown at 37°C in a humidified atmosphere in Dulbecco's minimal essential medium (high-glucose) containing Earle's salts and supplemented with 10% fetal bovine serum and antibiotics (penicillin [100 U/ml], Fungizone [0.25 µg/ml], and streptomycin [100 µg/ml]). All cell culture reagents were from Invitrogen (Carlsbad, CA).

Analysis of ACSL4 Protein Expression

Cells were grown in either 96-well or 24-well plates. After a wash with phosphate-buffered saline without calcium or magnesium, either 40 µl (96-well) or 200 µl (24-well) of sample buffer (10 mM Tris-HCl, 1 mM EDTA, 2.5% SDS, 5% β-mercaptoethanol, 0.01% bromophenol blue, pH 8.0) was added to the well. Samples were then heated to 95°C for 5 minutes. Electrophoresis was performed using the PhastGel System from GE Healthcare (Piscataway, NJ). Precast 7.5% acrylamide gels were used with SDS buffer strips. We subjected either 1 or 4 µl of individual samples to electrophoresis. We used Precision Plus protein standards from Bio-Rad (Hercules, CA) as molecular weight markers. After separation, the proteins were transferred to a polyvinylidene fluoride membrane (Hydrabond-P) using the PhastGel transfer apparatus. The membrane was then blocked with 5% milk in phosphate-buffered saline–Tween (0.1%) for 1 hour, followed by an overnight incubation with a 1:2000 dilution of affinity purified rabbit anti-ACSL4 antibody [6]. A 1:5000 dilution of goat antirabbit HRP secondary antibody was used for the final step. Signals were visualized using ECL-Plus chemiluminescence reagent. All immunoblot reagents were from GE Healthcare, with the exception of the antibody to β-actin, which was purchased from Cell Signaling Technologies (Danvers, MA). Quantitation of band densities was accomplished using the Quantity One program from Bio-Rad.

Quantitation of Relative Cell Number

Relative differences in cell number were quantitated using the Cell Titer 96 AQueous Reagent purchased from Promega (Madison, WI). Protocols used were as described by the manufacturer.

Small Interfering RNA–Mediated Knockdown of ACSL4

Cells were plated in T-25 flasks in complete medium lacking antibiotic and allowed to attach overnight. Cell densities at the start of the experiment were between 30% and 60%. Transfection of small interfering RNA (siRNA; either control or ACSL4-specific Smart Pool siRNA purchased from Dharmacon, Lafayette, CO) into cells was accomplished using Lipofectamine RNAiMAX (Invitrogen) according to the protocol recommended by the manufacturer. Transfections were carried out for 48 hours.

Bioinformatics

Expression data derived from Affymetrix arrays were obtained from the following public databases: Oncomine (<http://www.oncomine.org/>), Array Express (<http://www.ebi.ac.uk/>), and Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>). The data shown represent the base 2 logarithms of the original values.

Statistical Analyses

A Student's *t* test (two-tailed) was used to determine significance, unless otherwise noted.

Results

Expression of ACSL4 in Breast Tumor Samples and Cell Lines

A search of the Oncomine database [10] yielded 10 separate studies that reported overexpression of ACSL4 mRNA in human ER-negative breast tumor samples [11–20]. Figure 1A illustrates the results of these studies. For the study by Miller et al. [11], we analyzed the relationship between ER and ACSL4 mRNA expression levels. Figure 1B shows that there is a highly significant inverse correlation between expression levels of ER and ACSL4 mRNA ($P < .0001$). ACSL1, 5, and 6 were also overexpressed in ER-negative tumor samples, but associated *P* values were generally 10-fold higher. ACSL3 expression, however, was down-regulated in ER-negative tumors compared with ER-positive tumors in four separate studies [14,18,21,22] with $P < .001$.

To further investigate the relationship between ACSL4 mRNA levels and ER status, we analyzed microarray expression data reported for 50 human breast cancer cell lines [23]. Figure 2A illustrates results for expression of the five ACSL isoforms in these cell lines as a function of ER status. We found that ACSL4 mRNA expression was significantly higher in ER-negative cells ($P < .0001$), whereas expression of ACSL3 mRNA was significantly lower ($P = .015$). We detected no differences in expression of ACSL1, 5, or 6 as a function of ER status. Figure 2B illustrates the range of ACSL4 mRNA expression levels seen in the various cell lines.

Our next goal was to evaluate whether the observed differences in ACSL4 mRNA expression were recapitulated at the protein level, and whether these putative differences were an exclusive property of ACSL4. To accomplish this, we assessed the levels of ACSL1 and ACSL4 protein relative to those of β-actin, using immunoblot analyses of protein extracts isolated from ER-positive and ER-negative breast cancer cell lines (Figure 2C). We found that all the cell lines expressed detectable levels of ACSL1. These results were consistent with microarray studies that demonstrated expression of ACSL1 mRNA in these cell lines (Figure 2A). In addition, we found no correlation between ER status and ACSL1 protein expression levels (Figure 2C), a finding consistent with studies at the mRNA level (Figure 2A). However, with respect to ACSL4, only those cells with normalized mRNA expression values greater than 2.9

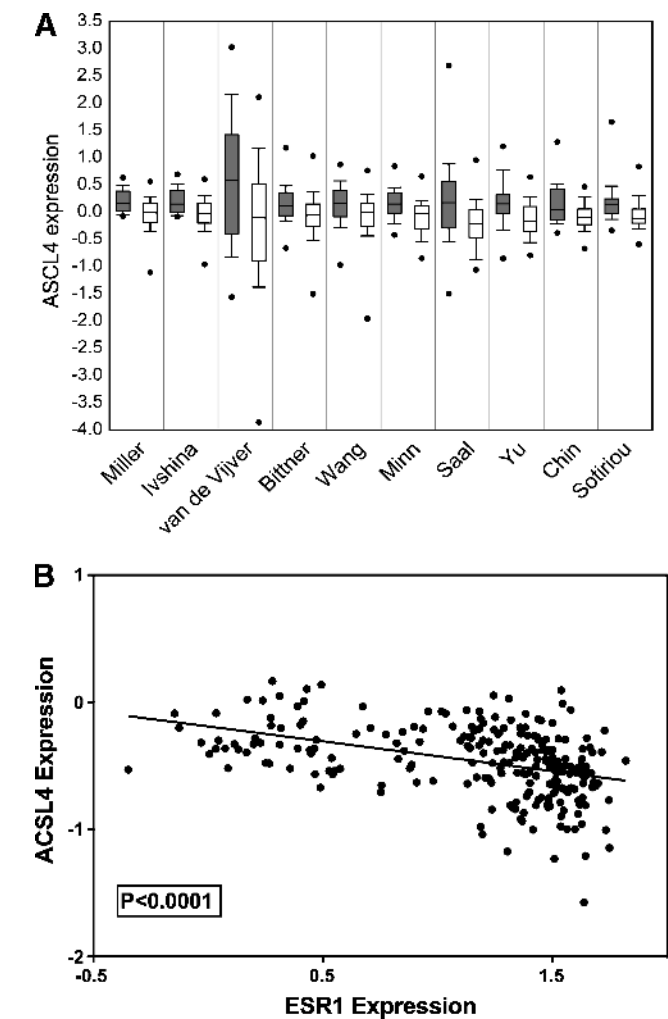


Figure 1. Expression of ACSL4 mRNA in human breast tumor samples. (A) Comparison of ACSL4 mRNA levels in ER-negative (solid bars) versus ER-positive human (open bars) breast tumors in ten independent gene expression profile data sets (denoted by study first author). Box-and-whisker plots indicate median, lower, and upper quartiles, and the smallest and largest values. Differences between ER-positive and ER-negative values are significant with $P < .001$ for all studies represented. Data provided by Oncomine as normalized expression units. (B) Relationship between ACSL4 expression and ESR1 (ER) expression in the study by Miller et al. [11]. The samples evaluated comprised 34 ER-negative and 213 ER-positive tumors. Data provided by Oncomine as normalized expression units.

appeared positive for ACSL4 by immunoblot analysis (Figure 2C). BT-20 cells had an intensity value of 4.55; MCF-7 cells, 2.52; MDA-MB-231 cells, 5.20; MDA-MB-415 cells, 3.14; SKBR3 cells, 2.77; and T47D cells, 2.62. Using the cutoff for expression indicated by the results from the immunoblot analyses, 17 (89%) of 19 ER-positive cell lines were negative for ACSL4 and 20 (65%) of 31 of ER-negative cells were positive for ACSL4 expression.

AR Status and ACSL4 Expression

A subset of mammary tumors known as molecular apocrine is ER-negative, AR-positive. Analysis of microarray expression data in this subset of breast cancers and in basal (ER-negative, AR-negative)

and luminal (ER-positive, AR-positive) breast tumors [24] revealed that ACSL4 mRNA levels were significantly lower in the molecular apocrine samples compared with the basal subset ($P < .001$, data not shown). Interestingly, of the 11 ER-negative cell lines that do not express ACSL4, 3 showed high levels of expression of AR mRNA. One of these cell lines, MDA-MB-453, has been shown to have a positive proliferative response to androgens [25]. To assess whether expression of ACSL4 and AR were inversely related, we first evaluated results from microarray studies in an ER-negative subset of tumors [14]. Interestingly, we found a significant inverse correlation between AR and ACSL4 mRNA expression levels (Figure 3A) reminiscent of that observed between ER and ACSL4. To explore this issue further,

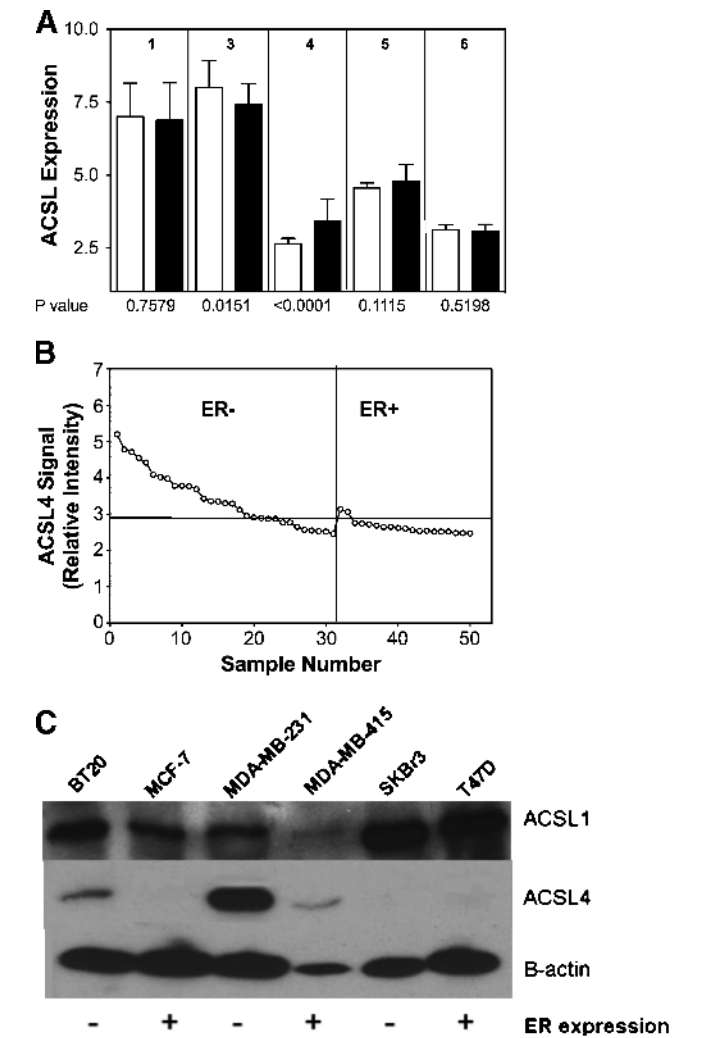


Figure 2. Effect of ER status on expression of ACSL4 in human breast cancer cell lines. Data provided by Array Express represents the log to the base 2 of the original data. (A) Expression levels of ACSL mRNA in human breast cancer cells. The expression data for 19 ER-positive (open bars) and 31 ER-negative (solid bars) breast cancer cell lines was compared for the five known isoforms of mammalian ACSL (1, 3, 4, 5, and 6). Values shown are the mean \pm SD. (B) Individual ACSL4 mRNA expression values for each cell line. The horizontal line indicates the cutoff, as determined by immunoblot, of ACSL4 positivity. (C) ACSL4 and ACSL1 protein expression in selected breast cancer cell lines. Immunoblot analyses were performed as described in Materials and Methods.

we compared AR and ACSL4 expression levels in prostate cancer cells. First, we analyzed data from a study that assessed mRNA expression in prostate cancer cell lines [26] and found that ACSL4 mRNA was overexpressed in AR-negative cell lines (data not shown). Second, to establish whether the differences in mRNA expression were recapitulated at the protein level, we assessed ACSL4 and β -actin expression in extracts from two AR-negative cell lines (PC3 and DU145), one AR-positive cell line (LNCaP), and one AR-positive cell line that is androgen-independent for growth (LNCaP-AI [9]). Figure 3B illustrates the results. We found that both of the AR-negative lines expressed high levels of ACSL4, whereas the AR-positive line did not express the protein. Importantly, we observed that loss of androgen sensitivity in LNCaP-AI cells was associated with increased expression of ACSL4, even in the presence of AR (Figure 3B). Finally, we analyzed results from an mRNA expression study in human prostate tumors [27] and found that ACSL4 levels were inversely correlated with AR expression (Figure 3C). These combined data strongly suggest negative links between steroid hormone receptor and ACSL4 expression that could reflect functional relationships in growth requirements or signaling events involving these proteins.

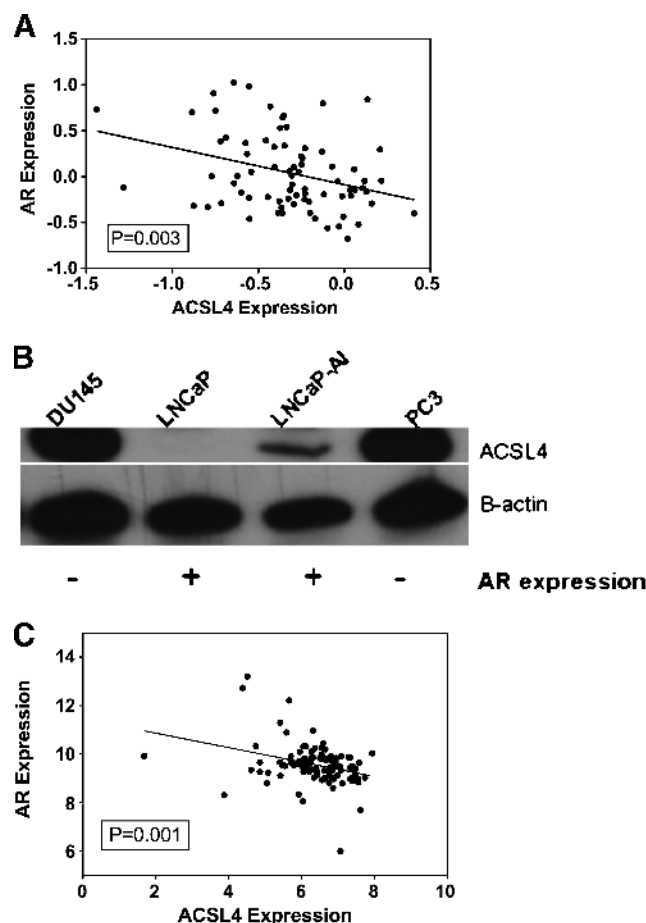


Figure 3. Effect of AR status on expression of ACSL4. (A) Relationship between AR and ACSL4 mRNA expression in 77 ER-negative breast tumor samples as reported in a study by Wang et al. [14]. Data provided by Oncomine. (B) Expression of ACSL4 protein in prostate cancer cell lines. Immunoblot analyses were performed as described in Materials and Methods. (C) Relationship between AR and ACSL4 mRNA levels in 98 human prostate tumors as reported by Holzbeierlein et al. [27]. Data provided by Gene Expression Omnibus.

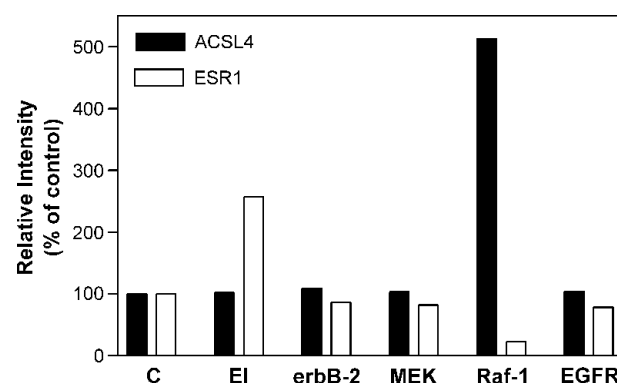


Figure 4. Induction of ACSL4 expression in MCF-7 cells constitutively expressing Raf-1. Data provided by Gene Expression Omnibus. C indicates control; EI, long-term E_2 -independent growth; *erbB-2*, cells overexpressing constitutively active c-*erbB-2*; MEK, cells overexpressing constitutively active MEK; *Raf-1*, cells overexpressing constitutively active Raf-1; *EGFR*, cells overexpressing ligand-activatable EGFR. Results shown are derived from the means of three separate determinations.

ACSL4 Expression in MCF-7 Cells Engineered to Overexpress Members of the Mitogen-Activated Protein Kinase (MAPK) Signaling Pathway

Our next goal was to start to identify intermediates that linked steroid hormone receptor and ACSL4 expression. Creighton et al. [28] previously reported that overexpression of key members of the mitogen-activated protein kinase (MAPK) signaling pathway in MCF-7 cells results in a molecular fingerprint characteristic of ER-negative human breast tumors. Our studies showed that MCF-7 cells, which express ER and are estrogen-dependent with respect to growth, express ACSL1 but do not express ACSL4 (Figure 2C). We hypothesized that genetic manipulation of MCF-7 cells leading to the expression of the ER-negative phenotype may also result in altered ACSL4 expression. To test this, we analyzed public microarray expression data sets from MCF-7 cells genetically manipulated to activate signaling through MAPK (Figure 4). We focused our attention on the expression of two genes, *ESR1* (probe 205225_at) and *ACSL4* (probe 202422_s_at), and found that transfection with constitutively active Raf-1 caused induction of ACSL4 ($P = .0009$; Figure 4). Overexpression of Raf-1 was also accompanied by loss of expression of ER mRNA ($P < .0001$), a finding consistent with a previous study showing loss of ER protein after transfection of MCF-7 cells with Raf-1 [29]. Interestingly, ACSL3 expression was downregulated in Raf-1-overexpressing cells (data not shown).

Effect of ACSL4 Ablation on MDA-MB-231 Cells

Because the evidence suggested that ACSL4 expression was associated with sex steroid hormone-independent growth, we wondered if ablation of this enzymatic activity would impact the ability of cells to proliferate. Clearly, the activity was not required for proliferation, in general, as evidenced by the ability of cells lacking ACSL4, such as MCF-7 and T47D, to grow quite well without it. A greater-than 95% reduction in ACSL4 protein expression was achieved by treating cells for 48 hours with ACSL4-specific siRNA, as shown in Figure 5A. The knockdown effect persisted for at least 3 days after removal of the transfection medium (data not shown). When proliferation of the knockdown cells was compared with that of control cells, no difference was observed (Figure 5B).

The ACSL inhibitor, triacsin C, is specific for inhibition of ACSL1, 3, and 4, with little or no effect on the activities of ACSL5 or 6 [30]. The half-maximal inhibitory concentration values reported for the three sensitive isoforms indicate that ACSL1 is the most sensitive, whereas ACSL4 is the least sensitive. In addition, this reagent has been demonstrated to inhibit proliferation and induce apoptosis in a variety of cancer cells [31]. When we compared the effect of triacsin C treatment on MDA-MB-231 control and knockdown cells, the results shown in Figure 5C were obtained. Ablation of cellular ACSL4 resulted in a three-fold increase in triacsin C sensitivity. The half-maximal inhibitory concentration for triacsin C was 1.59 μ M for control cells and 0.56 μ M for ACSL4 knockdown cells. These data suggest that ACSL4 activity makes a significant contribution to the overall ACSL activity required for growth and survival of cells.

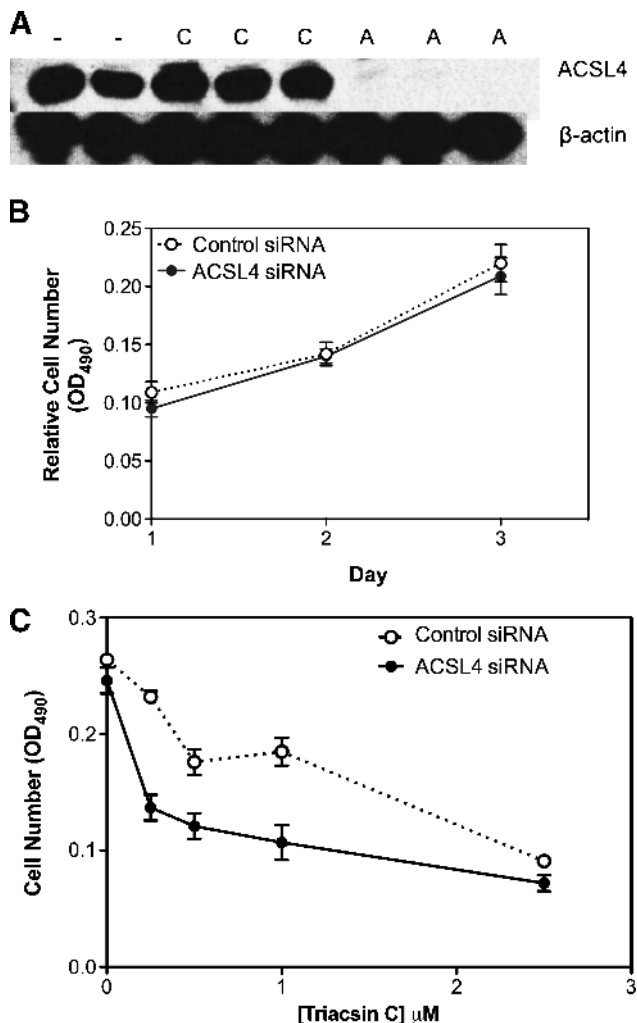


Figure 5. Effect of ablation of ACSL4 expression on proliferation of MDA-MB-231 cells. Cells were treated for 48 hours with either a control or ACSL4-specific siRNA as described in the text. Cells were then harvested and replated in multiwell plastic dishes and treated as described. (A) Immunoblot of cellular protein after a 48-hour transfection with no addition (–), control siRNA (C), or ACSL4-siRNA (A). Methods were as described in the text. (B) Growth curve of cells after 48 hours of treatment with control or ACSL4-siRNA. (C) Effects of triacsin C on relative cell number after 48 hours of treatment with control or ACSL4-siRNA. Relative cell numbers were assayed as described in the text. Values shown are means \pm SD.

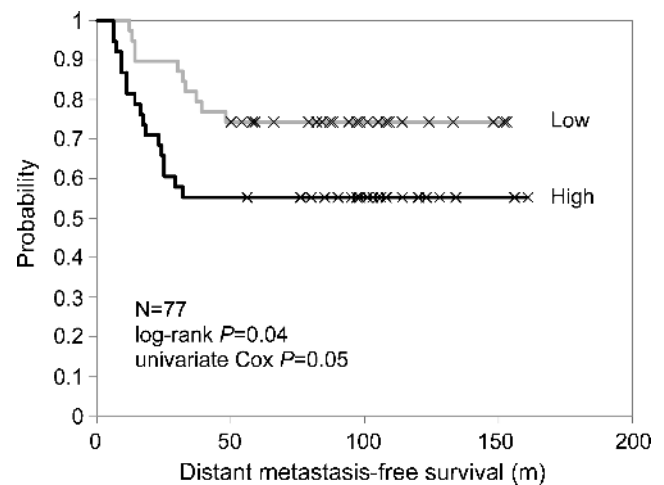


Figure 6. Correlation of ACSL4 mRNA levels with time to distant metastasis in ER-negative tumors. Kaplan-Meier analysis of 77 ER-negative tumors from node-negative patients who did not receive systematic adjuvant therapy. The log-rank test evaluates whether there are significant differences between tumors harboring high versus low ACSL4 levels. The univariate Cox test evaluates the association of ACSL4 with patient outcome, when the expression values are treated as a continuous variable (i.e., without making a cut). Data provided by Oncomine from a study by Wang et al. [14].

Prognostic Implication of ACSL4 Expression in ER-Negative Tumors

Because the expression of ACSL4 mRNA in ER-negative tumors varies from strong to none, we next investigated whether ACSL4 levels might have potential prognostic value. Using expression data from a study by Wang et al. [14], we correlated the level of expression of ACSL4 (high or low with respect to the median as cut point) with time of distant metastasis-free survival in 77 node-negative patients who did not receive systematic adjuvant therapy. The results of this analysis are shown in Figure 6. There was a significant difference between the groups ($P < .05$), suggesting that expression of ACSL4 may have prognostic value.

Discussion

Altered expression of lipid metabolic enzymes is a feature of a variety of cancers, including those that develop in breast tissues [32–34]. Both FASN and ACC have been shown to be essential for breast cancer cell survival [35], and inhibition of FASN activity has been shown to have potential chemopreventive [36] and therapeutic [37,38] applications. However, the precise role of altered lipid metabolism in the expression of the malignant phenotype has not been extensively studied. The reported increase in FASN activity in cancer cells may reflect high requirements of these proliferating cells for fatty acids; however, it is unclear whether these fatty acids are necessary for glycerolipid biosynthesis, used as an alternative energy source, or both. It has been reported that prostate cancer cells rely on fatty acids as an energy source [39], and several studies have found that blockade of FASN activity inhibits the growth of cancer cells [37]. A relationship between FASN expression and growth regulatory pathways has also been demonstrated [40].

The first step in the use of free fatty acids for either glycerolipid synthesis or β -oxidation is condensation with a molecule of CoA, a

reaction that is catalyzed by ACSLs. Thus far, five mammalian ACSLs that differ in subcellular location and substrate specificity have been identified [4]. The precise role of each isoform has not been delineated, but current evidence suggests that ACSL1 functions in hepatic glycerolipid synthesis [41,42], whereas ACSL3 and ACSL5 seem to increase β -oxidation of fatty acids in certain cells [43]. The localization of ACSL4 to peroxisomes suggests that this enzyme may function in fatty acid oxidation [44]. Inhibitor studies indicate that ACSL4 may also be involved in hepatic triacylglycerol synthesis [45]. Deletion of the human *ACSL4* gene has been associated with Alport syndrome, elliptocytosis, and mental retardation [46], and a mutated form of ACSL4 has been reported to be associated with X-linked mental retardation [47]. Mice heterozygous for ACSL4 deficiency present with abnormal uteri [48]. These studies indicate essential roles for ACSL4 in normal development and in reproduction.

It was previously shown that ACSL4 is overexpressed in colon and liver cancers [6,49,50], but to our knowledge, no studies in breast cancer have been reported to date. Comprehensive analyses of public expression databases indicated that ACSL4 mRNA expression is elevated in a subset of human breast cancer specimens. In addition, the data show that overexpression of ACSL4 occurs mainly in ER-negative tumors. We made similar observations in human breast cancer cell lines. Indeed, our analyses showed that 17 (89%) of 19 ER-positive cell lines were negative for ACSL4 expression, whereas 20 (65%) of 31 ER-negative lines expressed ACSL4 to varying degrees. Because a previous report indicated that changes in ACSL4 mRNA levels do not always reflect alterations in protein levels [51], we analyzed several breast cancer cell lines for ACSL4 protein expression. Our studies showed robust correlation between ACSL4 mRNA and protein expression, and they validated use of mRNA expression data sets as good indicators of ACSL4 protein expression. Our combined studies strongly suggested that ER status and ACSL4 protein levels were inversely correlated in human breast cancer.

The observation that several of the ER-negative, ACSL4-negative breast cell lines were positive for expression of AR mRNA led us to postulate that ACSL4 expression may also be linked to that of AR. The microarray data available for prostate tumor samples confirmed a negative correlation between ACSL4 expression and AR expression. To further examine this relationship, we analyzed ACSL4 expression in a variety of human prostate cancer cell lines and found that cells expressing AR lacked ACSL4, whereas those lacking AR expressed ACSL4. Interestingly, AR-positive prostate cancer cells that have developed the ability to grow in an androgen-independent fashion (LNCaP-AI) [52] now express ACSL4. Expression of ACSL3, conversely, has been reported to be stimulated by androgen treatment in prostate cancer [53]. Thus, we concluded that expression of ACSL4 was indicative of androgen insensitivity rather than simply absence of AR.

To further examine the relationship between hormone dependence and ACSL4 expression, we analyzed the consequences of loss of estrogen dependence in MCF-7 breast cancer cells. ER-positive MCF-7 cells in which the MAPK growth pathway is constitutively activated by overexpression of Raf-1 [28] have been shown to lose ER expression and exhibit an ER-negative molecular fingerprint. An examination of the microarray data accompanying these experiments indicates that Raf-1 overexpression and loss of ER are accompanied by induction of ACSL4 expression in these MCF-7 cells. Interestingly, constitutive activation of the MAPK pathway through overexpression of other elements of the pathway, such as EGFR or erbB2, did not result in induction of ACSL4 expression. It is important to emphasize that these correlative studies do not necessarily reflect causal functional relationships. How-

ever, an attractive possibility is that up-regulation of ACSL4 expression is necessary for hormone-independent growth of breast and/or prostate tumors. Studies along these lines could have important translational implications.

Another contribution of this study is related to our observations suggesting that ACSL4 levels may also have prognostic value. Approximately 10% of ER-positive tumors are unresponsive to hormone therapy, and many attempts have been made to identify a marker of ER sensitivity that complements ER expression data [54]. In general, expression of ER-inducible proteins has been considered a good index of estrogen sensitivity; however, this parameter may not always reflect hormone-dependent cellular growth. It is possible that simultaneous expression of both ER and ACSL4 might predict a lack of response to hormonal therapy, and we are currently undertaking further studies to evaluate this hypothesis.

With respect to ER-negative breast tumors, we found statistically significant differences in the time of distant metastasis-free survival in patient groups segregated based on ACSL4 mRNA expression levels. It is likely that these differences will become even more significant if protein levels are measured so that additional stratification can be made based on positive or negative protein expression status. Additional studies will be required to assess the utility of ACSL4 levels as prognostic biomarkers of breast cancer.

With respect to prostate cancer, it seems clear that the expression of ACSL4 is correlated with hormone-independent growth; however, the majority of prostate cancers are AR-positive, and it has been suggested that failure of a prostate tumor to respond to hormone-ablative therapy is most likely the result of increased sensitivity to androgens, rather than loss of sensitivity [55]. Thus, ACSL4 expression data would be of limited use.

The contribution of ACSL4 activity to the sex steroid receptor-negative phenotype of human breast cancer remains to be determined. There is evidence suggesting that ACSL3 plays a role in the response of prostate cancer to androgens [26,56], and that ACSL5 promotes glioma cell survival under extracellular acidosis conditions [57]. The induction of ACSL4 expression in sex steroid hormone-independent breast cancer cells suggests that this activity plays a role in facilitating hormone-independent growth, but the precise nature of this contribution remains to be determined. Results of knockdown studies presented here indicate that ACSL4 activity does not influence the growth or survival of MDA-MB-231 cells under the conditions used. It is possible that ACSL4 activity provides an advantage to cancer cells grown under more stringent conditions, such as low pH or hypoxia. But that ACSL4 activity plays a role in the lipid metabolism of MDA-MB-231 cells is indicated by the increased sensitivity of the cells to triacsin C after ablation of ACSL4 expression.

In summary, we have presented data that confirm an association between sex steroid hormone expression and ACSL4 expression in human breast and prostate cancer. More specifically, there is some evidence that ACSL4 expression is more closely associated with hormone-independent growth and, as such, may be a useful marker in determining response to hormonal therapy; however, much more work will be needed to validate such a conclusion.

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Long Chain Fatty Acyl-CoA Synthetase 4 Is a Biomarker for and Mediator of Hormone Resistance in Human Breast Cancer

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Abstract

The purpose of this study was to determine the role of long-chain fatty acyl-CoA synthetase 4 (ACSL4) in breast cancer. Public databases were utilized to analyze the relationship between ACSL4 mRNA expression and the presence of steroid hormone and human epidermal growth factor receptor 2 (HER2) in both breast cancer cell lines and tissue samples. In addition, cell lines were utilized to assess the consequences of either increased or decreased levels of ACSL4 expression. Proliferation, migration, anchorage-independent growth and apoptosis were used as biological end points. Effects on mRNA expression and signal transduction pathways were also monitored. A meta-analysis of public gene expression databases indicated that ACSL4 expression is positively correlated with a unique subtype of triple negative breast cancer (TNBC), characterized by the absence of androgen receptor (AR) and therefore referred to as quadruple negative breast cancer (QNBC). Results of experiments in breast cancer cell lines suggest that simultaneous expression of ACSL4 and a receptor is associated with hormone resistance. Forced expression of ACSL4 in ACSL4-negative, estrogen receptor α (ER)-positive MCF-7 cells resulted in increased growth, invasion and anchorage independent growth, as well as a loss of dependence on estrogen that was accompanied by a reduction in the levels of steroid hormone receptors. Sensitivity to tamoxifen, triacsin C and etoposide was also attenuated. Similarly, when HER2-positive, ACSL4-negative, SKBr3 breast cancer cells were induced to express ACSL4, the proliferation rate increased and the apoptotic effect of lapatinib was reduced. The growth stimulatory effect of ACSL4 expression was also observed *in vivo* in nude mice when MCF-7 control and ACSL4-expressing cells were utilized to induce tumors. Our data strongly suggest that ACSL4 can serve as both a biomarker for, and mediator of, an aggressive breast cancer phenotype.

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Introduction

Breast cancer is a heterogeneous disease comprised of distinct molecular subtypes that can be generally characterized by the expression status of receptors for estrogen (ER), progesterone (PR), human epidermal growth factor receptor 2

(HER2) and more recently, androgen (AR). Tumors that are negative for expression of ER and PR and for amplification of HER2 expression are termed triple negative breast cancers (TNBC) and display a more aggressive phenotype that is not amenable to steroid hormone/HER2-based targeted therapies and has a worse prognosis than receptor positive cancer [1].

Recent studies indicate that TNBC can be further stratified on the basis of expression of AR [2–4]. TNBC lacking AR are considered quadruple negative breast cancers (QNBC) and have been demonstrated to have a worse prognosis than TNBC in most studies [5], though not all [6].

We have previously demonstrated that the fatty acid metabolic enzyme, long chain fatty acyl-CoA synthetase 4 (ACSL4) is differentially expressed in human breast cancer samples as a function of expression of ER and AR [7]. ACSL4 is one of five isoforms of mammalian long chain acyl-CoA synthetases that activates fatty acids for further metabolism by condensing the fatty acid with a molecule of coenzyme A to form a thioester. Individual ACSL isoforms are characterized by their subcellular location and substrate specificity, although the significance of these characteristics has yet to be fully elucidated [8]. ACSL4 is unique in that it is localized to the peroxisome and the mitochondria-associated endoplasmic reticulum membrane, and has higher affinity for arachidonic acid (AA) and eicosapentanoic acid as substrates [9]. Previous studies have demonstrated that ACSL4 is overexpressed in both liver [10] and colon [11] cancer as well as in aggressive forms of breast cancer [7,12], and it has been suggested that metabolism of AA may play a role in mediating the effects of ACSL4 expression [12,13]. We showed that in both breast cancer cell lines and tumor samples, ACSL4 expression is inversely correlated with ER and AR levels. Importantly, in ER-negative tumors, high ACSL4 expression predicts a shorter time to distant metastases [7]. Thus ACSL4 serves as one of many biomarkers of an aggressive breast cancer phenotype and/or resistance to hormonal interventions. These data raise the question of the function of ACSL4 enzyme activity in mediating the aggressive phenotype associated with hormone independence. The investigation of the role of ACSL4 enzyme activity in mediating the aggressive phenotype associated with hormone independence may aid in the discovery of new therapeutic targets.

In the current study, we expand our previous findings to include a negative correlation between ACSL4 expression and HER2 amplification, and determine that ACSL4 levels correlate positively with the most aggressive QNBC. Furthermore, we demonstrate the impact of induced ACSL4 expression on cell growth, invasion and resistance to hormones.

Materials and Methods

Ethics Statement

Commercially available human cell lines and anonymous patient data from public databases were used for this study; as such, no patient consents were required and an exemption was granted by the Subcommittee for Human Studies of the Veterans' Administration Medical Center on 12/09/08. Approval for animal experiments was obtained from the Institutional Animal Care and Use Committee of NYU School of Medicine (NYU IACUC # 110506-02).

Tissue culture, cell proliferation and invasion assays

MDA-MB-231 cells, MCF-7 cells and SKBr3 cells (American Type Culture Collection) were maintained in DMEM (Life

Technologies) supplemented with 10% heat-inactivated bovine serum (fetal bovine serum), 1 U/ml of penicillin and 1 µg/ml of streptomycin. Cell proliferation was measured by the colorimetric WST assay under different growth conditions, in either complete medium, or phenol red-free medium containing charcoal-stripped with or without defined levels of estrogen (1 nM 17β- estradiol). Anchorage-independent cell growth in soft agar was performed in triplicate with cells (4×10^4) suspended in 2ml of medium containing 0.35% agar (Becton Dickinson) spread on top of 5ml of 0.7% solidified agar. Numbers and size of colonies were calculated.

Matrigel invasion assays were performed by adding 750 µL media with a chemoattractant (5% FBS) to the lower chamber of a BD Biocoat Matrigel Invasion Chamber (BD Bioscience, Bedford). A cell suspension (5×10^4) in 0.5 ml DMEM with 0.1% BSA was placed on the insert of the 24-well chamber. After 18 hours of incubation, the non-invading cells on the upper surface of the filter member were removed with a cotton swab. Invasive cells on the lower surface of the filter member were stained via Diff Quik stain and counted under light microscopy. The percentage of invasion was expressed as the ratio of invading cells over cell number normalized on day 2 of the growth curve. These methods have been previously described [14].

Transfection of MCF-7 and SKBr3 cells

To establish a stable cell line that conditionally expressed ACSL4, the MCF-7 Tet-On Advanced Cell Line (Clontech, CAT# 631153), grown in DMEM medium supplemented with 400 µg/ml G418, was subsequently transfected with either the pTRE-ACSL4 plasmid or the empty pTRE plasmid as a control. Individual cell clones were selected and isolated in the presence of 400 µg/ml G418 and 2 µg/ml puromycin in the culture medium. Single cell clones were cultured at high cell density in the presence and absence of 1 µg/ml doxycycline for 24 h and ACSL4 expression confirmed by immunoblot analyses.

A second method was utilized to force expression of ACSL4 in MCF-7 and SKBr3 cells. The Precision LentiORF-ACSL4 and RFP control viral particles (Thermo Scientific Company, CAT # OHS5833 and OHS5899) were incubated with MCF-7 and SKBr3 cells for 15 hours in high glucose DMEM containing 10% FBS, 10 µg/ml polybrene (Sigma) at 37°C in an atmosphere of 5% CO₂. On the next day, the infected cells were washed twice with DMEM medium and individual cell clones were selected and grown in the presence of 10 µg/ml Blasticidin S.

RNA extraction, semi-quantitative RT-PCR and siRNA knockdown

Total RNA was extracted from cells using the RNAqueous-4PCR kit (AM1914, Ambion). RetroScript kit (AM1710, Ambion) was used for cDNA synthesis with isolated RNA as template, according to manufacturer's instructions. 5 µl of the reverse transcription mixtures was used as template in 50 µl reactions. The PCR parameters were set as follow: 95°C -30 sec, 60°C-30 sec and 72°C- 30 sec. 15 µl of PCR product was separated on 2% agarose gel.

Small Interfering RNA-mediated knockdown of ACSL4 in MDA-MB-231 cells was performed in T-25 flasks in complete medium lacking antibiotic after cells were allowed to attach overnight. Cell densities at the start of the experiment were between 30% and 60%. Transfection of small interfering RNA (siRNA; either control or ACSL4-specific Smart Pool siRNA purchased from Dharmacon, Lafayette, CO) was accomplished using Lipofectamine RNAiMAX (Invitrogen) according to the protocol recommended by the manufacturer. Transfections were carried out for 48 hours.

Analysis of microarray data

Gene expression in MCF-7 and cells was analyzed with Affymetrix GeneChip Arrays. Three individual biological replicate samples of RNA were assayed for each experimental condition. The target populations and GeneChips were prepared, hybridized, and scanned according to the manufacturer's instruction. Briefly, 1 µg total RNA, isolated as described above, was reverse transcribed with a poly-(T) primer containing a T7 promoter, and the cDNA made double-stranded. An *in vitro* transcription was done to produce biotinylated cRNA, which was then hybridized to the GeneChips. The chips were washed and stained with streptavidin-conjugated phycoerythrin using an Affymetrix FS-450 fluidics station, and data was collected with Affymetrix GeneChip Scanner 3000.

Microarray expression data was processed by Robust multichip average (RMA) normalization by GenePattern in the ExpressionFileCreator module and/or GeneSpring GX11 software (Agilent). CEL files were transformed into GCT format with normalized probe set intensity values. Comparative Marker Selection was used to calculate p-value, FDR, FWER, and fold change to select statistical significant candidate probe sets. Gene Set Enrichment Analysis (GSEA) and NIH DAVID databases were used to interpret the ranked probe sets to identify significantly enriched biological processes and gene-based categories in the Gene Ontology, KEGG pathway and Reactome databases. Microarray data has been deposited at the Gene Expression Omnibus web site (GSE40968).

Immunoblot analysis

Immunoblot analysis was used to assess expression of ACSL4 and ER, with β -actin utilized as a loading control. Methods were as previously described [15]. In brief, the cells were lysed in an appropriate volume of lysis buffer containing protease cocktail inhibitor (P8340, Sigma) and the extracts were separated using SDS-polyacrylamide gel electrophoresis. The proteins were transferred to a PVDF nitrocellulose membrane for western blot analysis and developed with antibodies against ACSL4 (S0101, Epitomics), ER (SC-542, Santa Cruz), and β -actin (A5441, Sigma), and with the appropriate horseradish peroxidase-conjugated secondary antibody (7076S or 7074S, Cell Signaling). Protein bands were identified by imaging with a ChemiDoc XRS system. Densities were quantitated using the Quantify One 4.6.9 software system (Biorad).

Apoptosis assays

Control- and ACSL4-transfected cells were treated with tamoxifen, triacsin C, etoposide or lapatinib in complete medium for 72 hours. Apoptosis was measured using the Caspase-Glo assay kit (Promega, Madison USA). Briefly, after the plates containing cells were equilibrated at room temperature for 30 minutes, 100 µl of Caspase-Glo reagent was added to each well, the content of the well was gently mixed with a plate shaker at 300–500 rpm for 30 seconds followed by incubation at room temperature for 8 hours. The luminescence value of each sample was measured with luminometer (Thermo Labsystems) using 1 minute lag time and 0.5 second/well read time. The experiments were performed in triplicate and repeated on two separately-initiated cultures.

Proteomic Pathway Array Analysis

The analyses were carried out as previously described [16–19]. In brief, proteins were extracted from cells using a lysis buffer containing 20 mmol/L Tris-HCl (pH 7.5), 20 mmol/L sodium pyrophosphate, 40 mmol/L B-glycerophosphate, 30 mmol/L sodium fluoride, 2 mmol/L EGTA, 10 mmol/L NaCl, and 0.5% NP-40. The lysate was sonicated 3 times for 15 seconds each time, and then centrifuged (14,000 rpm, 30 minutes, and 4°C). The tubes were kept on ice throughout the process. The protein concentration was determined with the BCA Protein Assay Kit (PIERCE). Isolated proteins were separated by SDS-PAGE (10% acrylamide). Three hundred micrograms of protein extracts were loaded in a well across the entire width of gel for SDS-PAGE, followed by electro-transfer to a nitrocellulose membrane. The membrane was then blocked for 1 hour with 5% milk or 3% bovine serum albumin and clamped on to a Mini-PROTEAN II Multiscreen apparatus that isolates 20 channels across the membrane (Bio-Rad). Two or 3 antibodies were added to each channel and incubated overnight at 4°C. Different sets of antibodies were used for each membrane after stripping the previous set of antibodies. Antibodies were purchased either from Cell Signaling Technology, Inc., or from Santa Cruz Biotechnology, Inc. Two separate analyses were run for each sample. In each set, antibodies and protein levels were normalized by using β -actin and glyceraldehyde-3-phosphate dehydrogenase as standards. Chemiluminescence signals were captured by using the ChemiDoc XRS System. Differences in protein levels were determined by densitometric scanning and normalized to internal standards.

Xenograft studies in nude mice

Control-transfected and ACSL4-expressing MCF-7 cells were generated by stable transfection with lentiviral particles as described above. 1×10^6 cells from a single clone were mixed with Matrigel (Becton Dickinson) at a ratio of 1:1 and inoculated into the right inguinal mammary gland of 4- to 5-week-old female Nu/Nu BALB/c athymic nude mice (Frederick National Laboratory, NCI Animal Program). Both intact and ovariectomized animals were evaluated. The animals were given no exogenous estrogen. There were 10 animals for each experimental condition. The tumor growth was monitored and tumor volume measured every 3 days. The tumor volume was calculated as $l \times d \times h \times 0.52$ [20].

Table 1. Differential expression of ACSL4 in breast cancer as a function of receptor status.

Study	Sample Type	Target Group (no.)	Other Group (no.)	TG/OG	p value
Neve [21]	Cell lines	ER- (31)	ER+ (19)	1.71	8.00E-05
		TNBC (24)	Other (26)	1.79	7.00E-06
		QNBC (22)	Other (28)	1.88	3.75E-08
Hoefflich [22]	Cell lines	QNBC (22)	Other (29)	12.29	4.59E-08
Hess [23]	Tumor	TNBC (58)	Other (120)	1.33	6.70E-03
TGCA ¹	Tumor	TNBC (49)	Other (300)	1.54	7.30E-05
Wang [25]	Tumor	TNBC (55)	Other (200)	1.57	2.55E-10
Waddell [26]	Tumor	TNBC (22)	Other (44)	2.24	4.08E-06

Data was taken from arrays published by the authors. The p value, calculated using a two-tailed Student t-test, is for the difference in ACSL4 expression values between the designated groups. TG/OG is the ratio of the relative ACSL4 value for the Target Group divided by the Other Group. The number in parentheses denotes the sample size.

1. Data deposited at www.oncomine.com

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Statistical Analyses

Data was analyzed using the two-tailed Student t-test to compare means and the 2-way ANOVA test to compare growth curves. Differences were considered statistically significant for $p < 0.05$. For calculation of the predicative value of ACSL4 as a biomarker, a diagnostic test evaluation was carried out as described (http://www.medcalc.org/calc/diagnostic_test.php).

Results

QNBC express high levels of ACSL4

Publicly available microarray data was analyzed to determine the correlation between ACSL4 mRNA expression and expression of steroid hormone/HER2 receptors (ER, PR, AR and HER2). Results consistently revealed an inverse relationship between ACSL4 expression and receptor status. Table 1 illustrates this finding for several different studies of either cell lines [21,22] or tumor samples [23-26]. When one compares ACSL4 mRNA expression levels in ER-positive with that in ER-negative cells, the p value is 8.0E-05. Separating the samples by TNBC status decreases this p value by 10-fold, and adding AR-negative samples to the TNBC cohort, referred to as quadruple negative breast cancer (QNBC), further increases the significance of the difference. Utilizing expression array data from a different experiment where a total of 51 cell lines were analyzed, 21 of which were not included in the first analysis, the results for differential expression of ACSL4 versus QNBC yielded a p value of 4.59E-08. Thus two separate experiments yielded the same results.

Results were similar in studies of tumor samples also shown in Table 1. In evaluating ACSL4 mRNA levels in tumor samples, contamination with stromal and normal tissue must be considered. To determine the potential relevance of such contamination in evaluating ACSL4 levels, we determined ACSL4 expression levels in normal and stromal tissue utilizing microarray data reported for microdissected breast tumor tissue [27]. Results indicate that stromal tissue expresses high levels of ACSL4, while normal tissue expresses moderate levels (data not shown). This is not surprising since neither stromal tissue nor the majority of epithelial cells in normal

tissue expresses receptors [28]. Thus in evaluating the expression data for human tumor samples, contamination with stroma and normal tissue might explain the variability in results when compared with those seen for cloned breast cancer cell lines. Note also that the ratio of the relative ACSL4 values between the groups does not take into account that the lower relative values have been empirically determined to represent the absence of ACSL4 expression, as we have previously determined by immunoblot analysis [7]

Predictive value of ACSL4 as a biomarker for QNBC

Tables 2 and 3 detail the ACSL4 and receptor status of 71 individual cell lines. The ACSL4 status was determined for the cell lines by comparing the relative expression values to those previously validated by immunoblot [7]. The status for ER, PR and HER2 were derived from the relevant publications [21,22]. The status for AR was determined from microarray data. Table 2 lists cell lines that are positive for one or more receptors, while table 3 lists those cell lines that are QNBC. A statistical analysis was carried out to determine the utility of ACSL4 status (positive or negative) as a predictor of QNBC status. Table 4 summarizes the statistical data regarding the relationship between ACSL4 expression and QNBC status in these cell lines. The estimated value for sensitivity is 78% and for specificity is 86%. The positive and negative predictive values were not calculated because prevalence of QNBC in the cell lines was not comparable to that seen in breast cancer specimens. Of particular interest is the subset of cells that either co-express ACSL4 and a receptor or fail to express either ACSL4 or a receptor. These cell lines are listed in table 5. While it is possible that these represent false positives or false negatives, it is also possible that these cells comprise separate molecular subtypes (ACSL4+, non-QNBC and ACSL4-, QNBC) with implications for prognosis and treatment response. Thus addition of ACSL4 status as a biomarker might increase the predictive value of receptor status alone, and allow us to define a new category of effective QNBC for those specimens that express both ACSL4 and receptors. Those QNBC that fail to express ACSL4 might be designated as pseudo-QNBC.

Table 2. ACSL4 expression in steroid hormone/HER2 receptor positive breast cancer cell lines.

Cell Line	ACSL4	HER2	ER	PR	AR
600MPE	N	N	P	N	N
AU565	N	P	N	N	N
BT474	N	P	P	P	P
BT483	N	N	P	N	N
Cama1	N	N	P	N	P
EFM19	N	N	P	N	N
EFM192A	N	P	P	N	N
HCC1007	N	P	P	N	P
HCC1008	N	N	P	N	N
HCC1419	N	P	P	N	N
HCC1428	N	N	P	N	N
HCC1569	P	P	N	N	N
HCC1954	P	P	N	N	N
HCC202	N	P	N	N	P
HCC2218	N	P	N	N	P
KPL1	N	N	P	N	N
KPL4	N	P	P	N	P
LY2	N	N	P	N	N
MCF-7	N	N	P	P	N
MDA-134VI	N	N	P	N	P
MDA175VII	N	N	P	N	N
MDA361	N	N	P	P	N
MDA415	P	N	P	N	P
MDA453	N	N	N	N	P
MDA468	N	N	N	N	N
MFM223	N	N	N	N	P
SKBr3	N	P	N	N	N
SUM185PE	N	N	N	N	P
SUM190PT	P	P	N	N	N
SUM225CWN	N	P	N	N	N
SUM44PE	N	N	P	N	P
SUM52PE	N	N	P	N	N
T47D	N	N	P	P	N
UACC812	N	P	P	N	N
UACC893	N	P	N	N	P
ZR75-1	N	N	P	N	P
ZR75-30	N	P	P	N	P
ZR75B	P	N	P	N	N

Data for ER, PR, HER2 and AR were derived as described in the text from public databases. ACSL4 status was determined based the correlation between expression data and representative immunoblot data previously published [7]. P=positive; N=negative

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Relationship of ACSL4 expression to intrinsic molecular subtype

Although receptor status generally aligns with molecular subtype, that is not always the case [29]. For example, around 20% of TNBC are non-basal-like while 30% of basal-like tumors are non-TNBC. Using molecular subtype characterizations previously described for 52 breast cancer cell lines [30], as well as receptor status described by Neve et al [21] for the same cell lines, we examined ACSL4 status as a function of molecular subtype as shown in Figure 1. Figure 1A

details the receptor status and ACSL4 status for each individual cell line, while 1B indicates the range, mean and standard deviation of ACSL4 values as a function of molecular subtype.

A similar correlation is seen with respect to ACSL4 mRNA expression in human tumor samples. In a series of tumor samples comprised of 5 classified as basal-like, 6 classified as ERBB2-enriched and 16 classified as luminal [22], ACSL4 mRNA expression differed among the subtypes as follows: luminal, 317 ± 130 , basal-like, 565 ± 128 , and ERBB2-enriched, 364 ± 83 . The difference between ACSL4 mRNA

Table 3. ACSL4 expression in steroid hormone/HER2 receptor negative breast cancer cell lines.

Cell Line	ACSL4	HER2	ER	PR	AR
BT-20	P	N	N	N	N
BT549	P	N	N	N	N
CAL120	P	N	N	N	N
CAL148	N	N	N	N	N
CAL51	N	N	N	N	N
CAL85-1	P	N	N	N	N
DU4475	P	N	N	N	N
EVSA-T	N	N	N	N	N
HCC1143	N	N	N	N	N
HCC1187	N	N	N	N	N
HCC1395	P	N	N	N	N
HCC1500	P	N	N	N	N
HCC1599	P	N	N	N	N
HCC1806	N	N	N	N	N
HCC1937	P	N	N	N	N
HCC2157	P	N	N	N	N
HCC2185	N	N	N	N	N
HCC3153	P	N	N	N	N
HCC38	P	N	N	N	N
HCC70	P	N	N	N	N
HDQ-P1	P	N	N	N	N
HS578T	P	N	N	N	N
JIMT1	P	N	N	N	N
MCF10A	P	N	N	N	N
MCF12A	P	N	N	N	N
MDA231	P	N	N	N	N
MDA436	P	N	N	N	N
MDAMB157	P	N	N	N	N
MX1	P	N	N	N	N
SUM1315	P	N	N	N	N
SUM149PT	P	N	N	N	N
SUM159PT	P	N	N	N	N
SW527	P	N	N	N	N

Source of data and abbreviations as described for Table 2.

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expression in luminal and basal-like tumors was significant ($p=0.001$) as was that between ERBB2 and basal-like ($p=0.011$). There was no significant difference between expression in luminal and ERBB2-enriched ($p=0.417$). By contrast, although the trend was the same, there was no significant difference between the subtypes for expression of CD44, generally considered a marker for claudin-low and basal-like subtypes.

ACSL4 promotes growth of ER-positive breast cancer cells *in vitro*

As we have previously demonstrated, neither ER-positive MCF-7 nor HER2-positive SKBr3 cells normally express ACSL4 as assessed by immunoblot [7]. Utilizing a doxycycline-dependent conditional expression system, the effect of ACSL4 expression on MCF-7 cells was examined. When compared

with control cells, ACSL4-expressing MCF-7 cells exhibited an increased rate of growth (Figure 2C). In addition, ACSL4-expressing MCF-7 cells no longer responded to estradiol treatment with an increase in growth (Figure 2D), although an increase was seen in control-transfected MCF-7 cells (Figure 2E). In fact, a decrease was observed in the presence of estradiol. This loss of estrogen dependent growth was accompanied by a decreased expression of ER, PR and AR (Figure 2A and 2B).

ACSL4 expression also increased anchorage-independent growth of MCF-7 cells. As demonstrated in Figure 3, both the number and size of the colonies increased significantly (Figure 3A and 3B). In addition to ACSL4's effect on anchorage-independent growth of ACSL4-expressing MCF-7 cells, invasion capability, as measured by BD matrigel invasion assays, was also increased as shown in Figure 4 A-C. Consistent with this finding, when ACSL4-positive, QNBC

Table 4. Predictive value of ACSL4 as a marker for QNBC in breast cancer cell lines.

	Estimated Value	95% Confidence Interval	
		Lower Limit	Upper Limit
Sensitivity	0.787879	0.606013	0.903687
Specificity	0.868421	0.711162	0.950527
Positive Likelihood Ratio	5.99	4.38	8.19
Negative Likelihood Ratio	0.24	0.13	0.47

Data was derived using the ACSL4 status for the cell lines indicated in tables 2 and 3. In a total of 71 cell lines, 31 are QNBC and 40 are positive for one or more receptor. Of the QNBC cell lines, 26 are positive for ACSL4 and 5 are negative. Of the receptor-positive cell lines, 7 are positive for ACSL4 and 33 are negative.

Sensitivity: probability that ACSL4 will be positive when QNBC is present (true positive rate).

Specificity: probability that ACSL4 will be negative when QNBC is not present (true negative rate).

Positive likelihood ratio: ratio between the probability of a positive ACSL4 result given the presence of QNBC and the probability of a positive ACSL4 given the absence of QNBC, i.e.

= True positive rate / False positive rate = Sensitivity / (1Specificity)

Negative likelihood ratio: ratio between the probability of a negative ACSL4 result given the presence of QNBC and the probability of a negative ACSL4 result given the absence of QNBC, i.e.

= False negative rate / True negative rate = (1Sensitivity)/ Specificity

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Table 5. Cell lines that are anomalies with respect to ACSL4 expression.

Cell line	ACSL4	ER	PR	HER2	AR
HCC1569	P	N	N	P	N
HCC1954	P	N	N	P	N
MDA415	P	P	N	N	P
SUM190PT	P	N	N	P	N
ZR75B	P	P	N	N	N
CAL148	N	N	N	N	N
CAL51	N	N	N	N	N
EVSA-T	N	N	N	N	N
HCC1143	N	N	N	N	N
HCC1187	N	N	N	N	N
HCC1806	N	N	N	N	N
HCC2185	N	N	N	N	N

Source of data and abbreviations as described for Table 2.

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MDA-MB-231 cells were treated with ACSL4 siRNA, as previously described [7], invasion capability was diminished (Figure 4 D-F).

Since forced ACSL4 expression reduces expression of ER in MCF-7 cells, we speculated whether the reverse would be true, that is, whether reduction of ER would impact ACSL4 expression. Figure 5 details results derived from mRNA expression data as previously reported [31]. In this report the authors treated MCF-7 cells with siRNA directed against ER and subsequently carried out microarray studies to assess alterations in gene expression resulting from the decrease in ER. ACSL4 mRNA expression is increased as a result of the decrease in ER, while there is no significant effect on either ACSL3 or ACSL6. ACSL1 is decreased, while ACSL5 is also increased. The effects seen on ACSL1 and ACSL5 consequent to ER ablation are not observed when comparing ACSL expression in breast cancer cell lines as a function of ER

expression, as we have previously reported [7]. However, an inverse relationship between ACSL5 and ER status was observed in our previous analysis of tumor sample data [7], although the results were not as significant as those we reported for ACSL4.

ACSL4 enhances tumor growth of ER-positive breast cancer cells *in vivo*

To determine whether the growth regulatory effects of ACSL4 expression in MCF-7 cells could be observed *in vivo*, we performed orthotopic intramammary tumor xenograft experiments using intact and ovariectomized nude mice. The results shown in Figure 6 reveal there is an increased rate of tumor growth in xenografts overexpressing ACSL4 (n=10) compared to vector control (n=10) (p=0.0118) consistent with the data derived from cell culture experiments. This effect of ACSL4 expression on tumor proliferation was observed in both

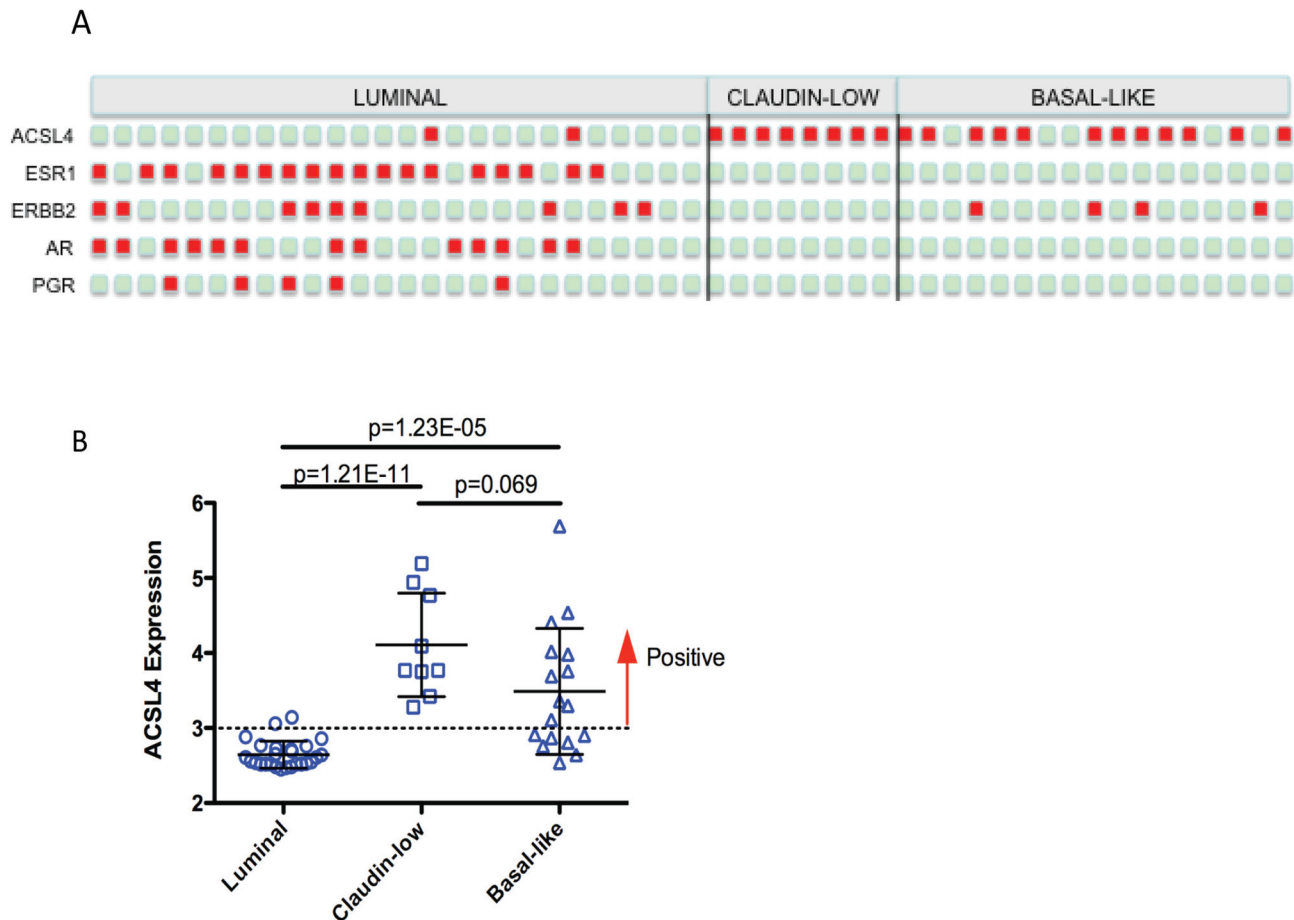


Figure 1. ACSL4 expression as a function of intrinsic molecular subtype. Microarray expression data and subtype allocations of 52 human breast cancer cell lines were obtained from previously published data [21,30]. Panel A lists expression status for ACSL4, ER (ESR1), HER2 (ERBB2), AR and PR (PGR). Green squares are negative for expression and red squares are positive. Identification of individual cell lines can be found in reference 30 (note: HBL-100, included in reference 30, was not included in this figure). Panel B shows ACSL4 mRNA relative expression values as a function of the molecular subtype of individual cell lines. Means and 1SD are shown, as well as the p values for the differences between subtypes.

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intact and ovariectomized mice. Immunohistochemical analysis of tumor samples indicated a significant increase in Ki67 staining in samples derived from ACSL4-expressing cells when compared to controls (Figure 6C-E).

ACSL4 promotes proliferation of HER2-positive breast cancer cells

Data presented in table 1 indicates that ACSL4 expression is inversely correlated with that of HER2 overexpression. We examined whether simultaneous expression of HER2 and ACSL4 might impact cell growth in a manner similar to that seen for simultaneous expression of ER and ACSL4. To test this, the HER2-positive, ACSL4-negative cell line, SKBr3, was stably transfected with ACSL4 cDNA using a lentivirus vector, and the effects on growth monitored. Figure 7 illustrates that ACSL4 expression results in a modest increase in growth compared with control-transfected cells. The same effect was

observed in medium containing complete (7A) as well as charcoal-stripped serum, with (7C) or without (7B) added estrogen.

ACSL4 expression impacts sensitivity to targeted as well as traditional treatment reagents

The loss of estrogen's stimulatory effect as a result of ACSL4 expression as shown above suggested that ACSL4-expressing MCF-7 cells might be less sensitive to tamoxifen treatment than control cells. To test this hypothesis, the response of control and ACSL4-MCF-7 cells to various treatments was compared. Figure 8 (A-C) documents the differential effects of tamoxifen, etoposide and the ACSL inhibitor, triacsin C, on control- and ACSL4-MCF-7 cells. In each case, expression of ACSL4 correlates with increased resistance to the therapeutic regimen, suggesting an overall effect to increase survival in general. These findings are in line with those previously

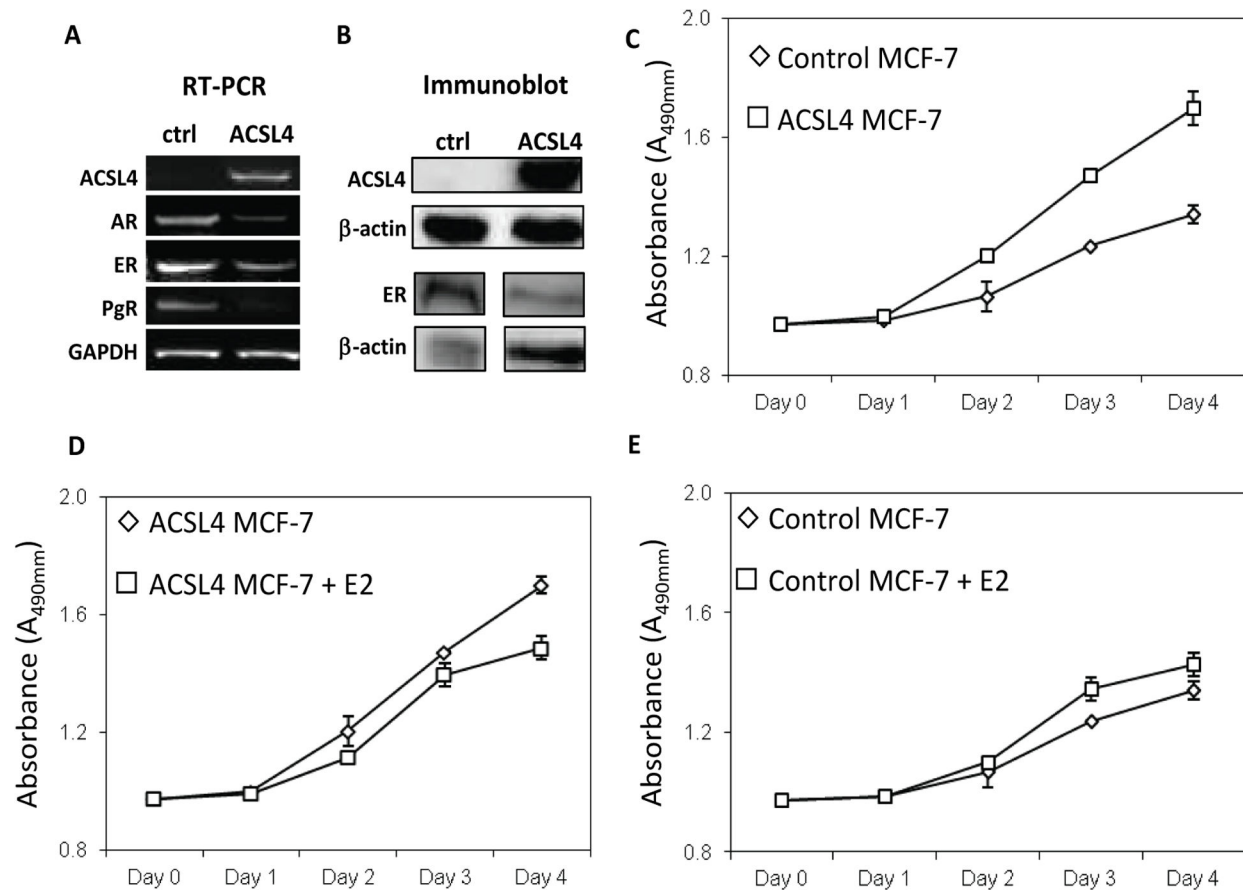


Figure 2. Effect of ACSL4 expression on breast cancer growth. A doxycycline-inducible line of MCF-7 cells was transfected with either a control or ACSL4-expressing plasmid as described in the text. Both control and ACSL4-MCF-7 cells were treated with 1μg/ml of doxycycline. (A) RT-PCR analysis of mRNA for ACSL4, AR, ER and PgR in vector control and ACSL4-MCF-7 cells. GAPDH was used as a loading control. (B) Immunoblot analysis of whole cell lysates showing expression of ACSL4 and ER with β-actin as loading control. The increase in ACSL4 is 163 fold, and the decrease in ER is 68%. (C) Comparison of proliferation of control with ACSL4-MCF-7 cells grown in phenol red-free medium supplemented with charcoal-stripped FBS. Values shown are the means of triplicate determinations ± 1SD. The difference between the curves is significant, $p < 0.0001$. (D) The effect of estradiol on proliferation of doxycycline-induced ACSL4-transfected MCF-7 cells grown as in (C). Values shown are the means of triplicate determinations ± 1SD. The difference between the curves is significant, $p < 0.0001$. (E) The effect of estradiol on proliferation of doxycycline-treated control MCF-7 cells grown as described above for panel C. Values shown are the means of triplicate determinations ± 1SD. The difference between the curves is significant, $p < 0.0001$.

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reported suggesting that ACSLs, in general, are cancer survival factors that can inhibit the efficacy of etoposide [32]

SKBr3 cells are normally sensitive to treatment with reagents that target HER2 activity. We tested the effect of ACSL4 expression on sensitivity to one of these reagents, lapatinib. Figure 8D demonstrates that ACSL4 expression induces some resistance to lapatinib as evidenced by decreased apoptosis in the lapatinib-treated SKBr3 cells that express ACSL4.

Impact of ACSL4 expression on down stream mRNA and protein expression

We next evaluated gene expression as a function of ACSL4 expression using both integrated Affymetric microarray analysis and proteomic pathway array analysis (PPAA) comparing control and ACSL4-transfected MCF-7 and SKBr3 cells. For the Affymetric microarray analysis, we assessed 1) the impact of acute, conditional expression of ACSL4 in MCF-7 cells using a tetracycline inducible model as well as 2) the impact of long-term ACSL4 expression in MCF-7 cells stably expressing ACSL4; and 3) the impact of long-term ACSL4 expression in SKBr3 cells stably expressing ACSL4. Note that the mRNA

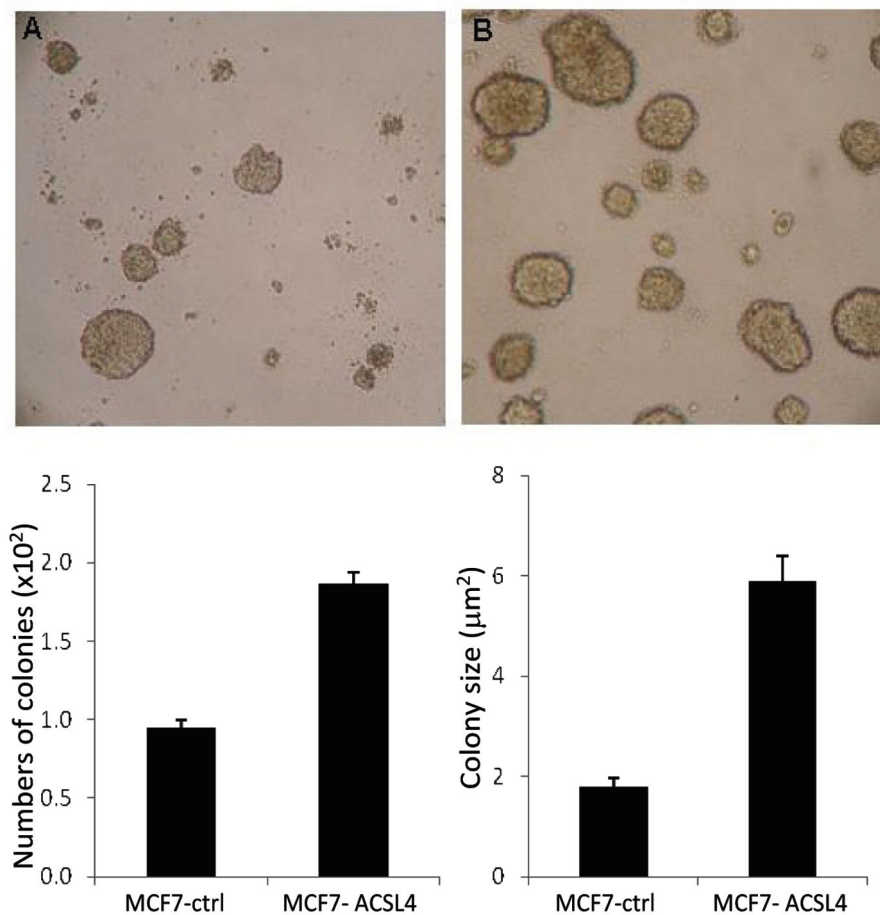


Figure 3. Effect of ACSL4 expression on anchorage-independent growth of MCF-7 cells. MCF-7 cells were stably transfected with ACSL4 cDNA utilizing a lentivirus vector, and control- and ACSL4-transfected cells compared with respect to anchorage-independent growth as described in the text. Panel A, shows control cells, and panel B are ACSL4-expressing cells. Panel C quantitates the number of colonies and panel D, the size of the colonies. Values shown in (C) and (D) are the means of triplicate determinations \pm 1SD. The differences shown are significant: For C, $p = 0.028$ and for D, $p = 0.009$.

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expression values for ACSL4 in the transfected cells are not increased due to the fact that the probe is directed against an untranslated region of the mRNA that was not present in the transfected cDNA. Table S1 is a list of genes affected by ACSL4 expression that were common across experiments. Tables 6, 7 and 8 list those genes whose expression was increased or decreased by at least 2-fold. Only one gene satisfied these criteria across all three experiments (table 6), autism susceptibility candidate 2 (AUTS2). Additional common genes were observed when comparing acute and stable ACSL4 expression in MCF-7 cells (table 7) and between MCF-7 and SKBr3 cells (table 8). Figure 9A validates the microarray data with respect to AUTS2 mRNA expression in both MCF-7 and SKBr3 cells. The precise function of AUTS2 is unknown; however mutations in this gene have been associated with neurodevelopmental disorders [33], as have mutations in ACSL4, which have been implicated in both X-linked mental retardation [34] and autism [35]. In order to further

analyze the inverse relationship between AUTS2 and ACSL4, we assessed the effect of inducing ACSL4 expression in MCF-7 cells by an alternate route. We have previously reported that constitutive expression of RAF-1 in MCF-7 cells causes induction of ACSL4 mRNA [7], and in the present study we report that ACSL4 is induced in MCF-7 cells as a result of siRNA-induced ablation of ER (Figure 5). Figure 9B shows the effect of these manipulations on AUTS2 mRNA expression. In both cases AUTS2 mRNA expression is decreased by 90% and 88% respectively.

An analysis of pathways common to all three experiments (table S2) indicates that ACSL4 expression results in the down regulation of a number of signal transduction pathways, including those involving both steroid and peptide hormones and growth factors, which supports the notion that ACSL4 expression induces hormone/growth factor resistance. In addition, pathways involving cytoskeletal organization and cell adhesion are also down regulated, as might be expected in

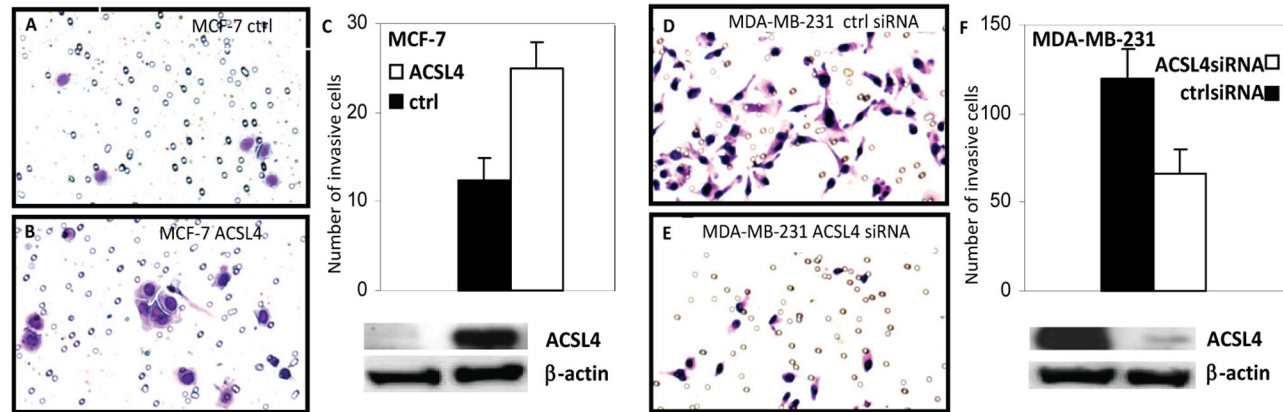


Figure 4. Effect of ACSL4 expression on invasion capability of breast cancer cells. MCF-7 cells were stably transfected with ACSL4 cDNA utilizing a lentivirus vector, and control- and ACSL4-transfected cells compared with respect to invasive potential as described in the text. Panel A, control MCF-7, B, ACSL4-MCF-7, C, bar graph indicating the average number of cells per field in 3 separate chambers ($p=0.005$), and an immunoblot blot analysis of ACSL4 expression in control and ACSL4- MCF-7 cells. For panels D, E and F, MDA-MB-231 were treated with either control or ACSL4 siRNA (20nM), and control and experimental cells were compared with respect to invasive potential as described in the text. Panel D, control siRNA-treated cells, E, ACSL4 siRNA-treated cells, F, bar graph indicating the average number of cells per field in 3 separate chambers ($p=0.005$), and an immunoblot blot analysis of ACSL4 expression in control siRNA and ACSL4 siRNA-treated cells.

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light of the observed effect of ACSL4 expression on invasion. Also consistent with a more aggressive phenotype is a reduction in the expression of genes involved in apoptosis. The ability of ACSL4 to increase proliferation is reflected in the upregulation of pathways that function in general cellular metabolism and DNA and RNA synthesis.

Results from PPAA analysis comparing control- and ACSL4-expressing MCF-7 and SKBr3 cells are shown in table 9. The pathways that appear activated by expression of ACSL4 in both cell lines are those involving PKC α / β II (Thr 638/641), L-selectin and alcohol dehydrogenase (ADH). In SKBr3 cells β -catenin levels were greatly increased. Figure 9C validates the increases seen in phospho-PKC α and β -catenin suggested by the PPAA results. The increase in β -catenin in SKBr3 cells is concomitant with an increased nuclear localization, as illustrated by the immunofluorescence study shown in Figure 9D. A role for the WNT signaling pathway in breast cancer progression has been suggested by data illustrating that loss of the WNT negative regulator, sFRP1, is associated with breast cancer progression and poor prognosis [36]. When ACSL4-positive MDA-MB-231 cells are forced to express sFRP1, ACSL4 mRNA expression decreases by 42% ($p = 0.003$), as illustrated in microarray data reported by Matsuda et al [37]. This data is deposited on the GEO website, GSE13806.

Methods were as described in the text and results shown are from two separate experiments. Fold change = ACSL4-transfected cells/control cells NC = no change; NE = not expressed

Discussion

Breast cancer is a heterogeneous disease in which treatment is complicated by varying degrees of steroid hormone/HER2 sensitivity and the development of resistance to therapies targeting the actions of these hormones and growth factors. The ability to characterize individual cancers as to their likelihood of responding to such therapies currently relies on the measurement of steroid hormone/HER2 receptors, their presence being indicative of a probable response to a therapy designed to block their action. However, *de novo* and acquired resistance to targeted therapies remains a frequent problem in hormone receptor-expressing as well as Her2 positive breast cancers [38,39]. We have presented evidence here that expression of a lipid metabolic enzyme, ACSL4, inversely correlates with the presence of steroid hormone and growth factor receptors in breast cancer, and as such may be a marker for the highly aggressive QNBC. The addition of AR to the triple negative characterization is supported by a variety of reports suggesting that TNBC that lack AR have a worse prognosis than AR-positive TNBC [5,40-44]. This inverse correlation between ACSL4 status and receptor status was significant for both studies in cell lines as well as tumor samples.

In order to determine whether ACSL4 status could serve as a biomarker for QNBC, we surveyed ACSL4 expression in 71 different cell lines and correlated the data with that for receptor expression. ACSL4 status predicted QNBC status with a sensitivity of 78% and a specificity of 86% (table 4). Of potential interest are those instances where either both ACSL4 and receptors are co-expressed or neither ACSL4 nor receptors are expressed (table 5). These data suggest the possibility of a further stratification of both receptor-positive

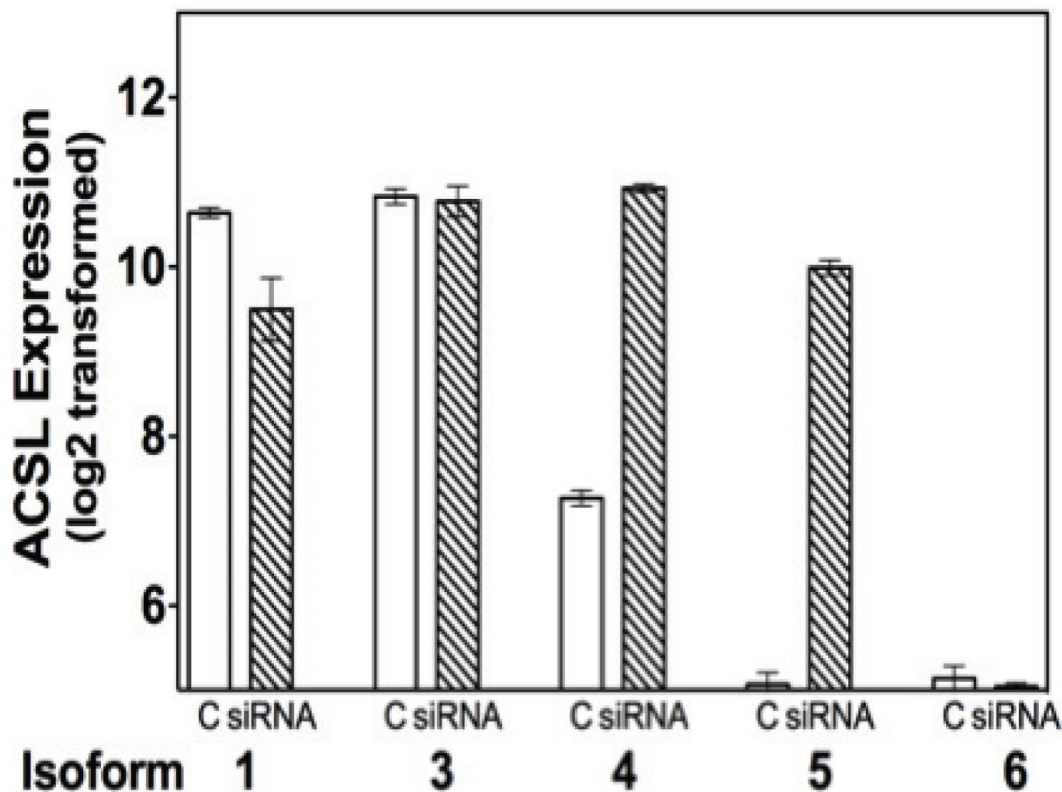


Figure 5. Effect of ablation of ER α on ACSL4 expression in MCF-7 cells. siRNA was utilized to mediate the silencing of ER expression in MCF-7 cells as described in reference [31]. The results shown are taken from an Affymetrix Human Genome U133 plus 2.0 Gene Chip microarray study reported on the Gene Expression Omnibus (GDS40610). Values shown represent the means \pm 1SD of triplicate determinations. The significance of the differences for isoform 1, $p=4.53E-03$, for isoform 4, $p=5.02E-07$, and for isoform 5, $p=9.67E-07$. No significant difference is observed for isoforms 3 and 6.

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breast cancer and QNBC based on ACSL4 status that might predict prognosis and/or response to therapy.

Stratifications have also been proposed based on intrinsic molecular subtype as defined by a variety of gene expression paradigms [29,45]. ACSL4 status is clearly associated with claudin-low, and to a lesser extent, basal-like breast cancer (Figure 1). There does not appear to be an association between ACSL4 status with respect to luminal subtypes [46]. This brings up the question of why ACSL4 has not been identified in any previous gene signature of molecular subtypes. We would suggest the possibility that the low level of relative expression values seen in positive samples as compared with negative samples underestimates the actual change observed in ACSL4 expression as a function of molecular subtype and receptor status, and thus ACSL4 is rejected as a function of the application of algorithms designed to capture the most significant differences. However, immunoblot data confirms that a log base 2 value of 3.5 can represent strong expression while a value of 2.5 is negative [7].

In support of ACSL4 functioning as a biomarker for effective QNBC, the data detailed here indicate that forced ACSL4 expression is capable of inducing resistance to hormone-

stimulated growth as well as reducing sensitivity to targeted therapies. In estrogen sensitive MCF-7 cells, expression of ACSL4 confers insensitivity to estrogen treatment with respect to growth as well as increased invasion capability (figures 2-4). In fact, there is a slight inhibition of growth in the presence of estrogen. These effects are accompanied by a reduction in expression of ER, PR and AR (Figure 2). ACSL4 expression in the absence of any hormonal treatment increases the growth rate of the cells in both complete serum (data not shown) as well as in charcoal-stripped serum. Similar results have been recently reported for ACSL4-expressing MCF-7 cells both *in vitro* and *in vivo* [47]. Reduction of ACSL4 levels in ACSL4-positive, QNBC, and MDA-MB-231 cells significantly inhibits invasion capability (Figure 4), confirming previous data from Maloberti et al [12]. Lastly, ACSL4 expression decreases apoptosis in MCF-7 cells in response to treatment with tamoxifen (ER antagonist), triacsin C (ACSL1, 3 and 4 antagonist) or etoposide (Figure 8). Thus the ability of ACSL4 to enhance survival is not limited to effects on estrogen action, but appears also to have a general overall effect. We have previously reported that attenuation of ACSL4 expression in the QNBC cell line, MDA-MB-231, causes increased sensitivity to

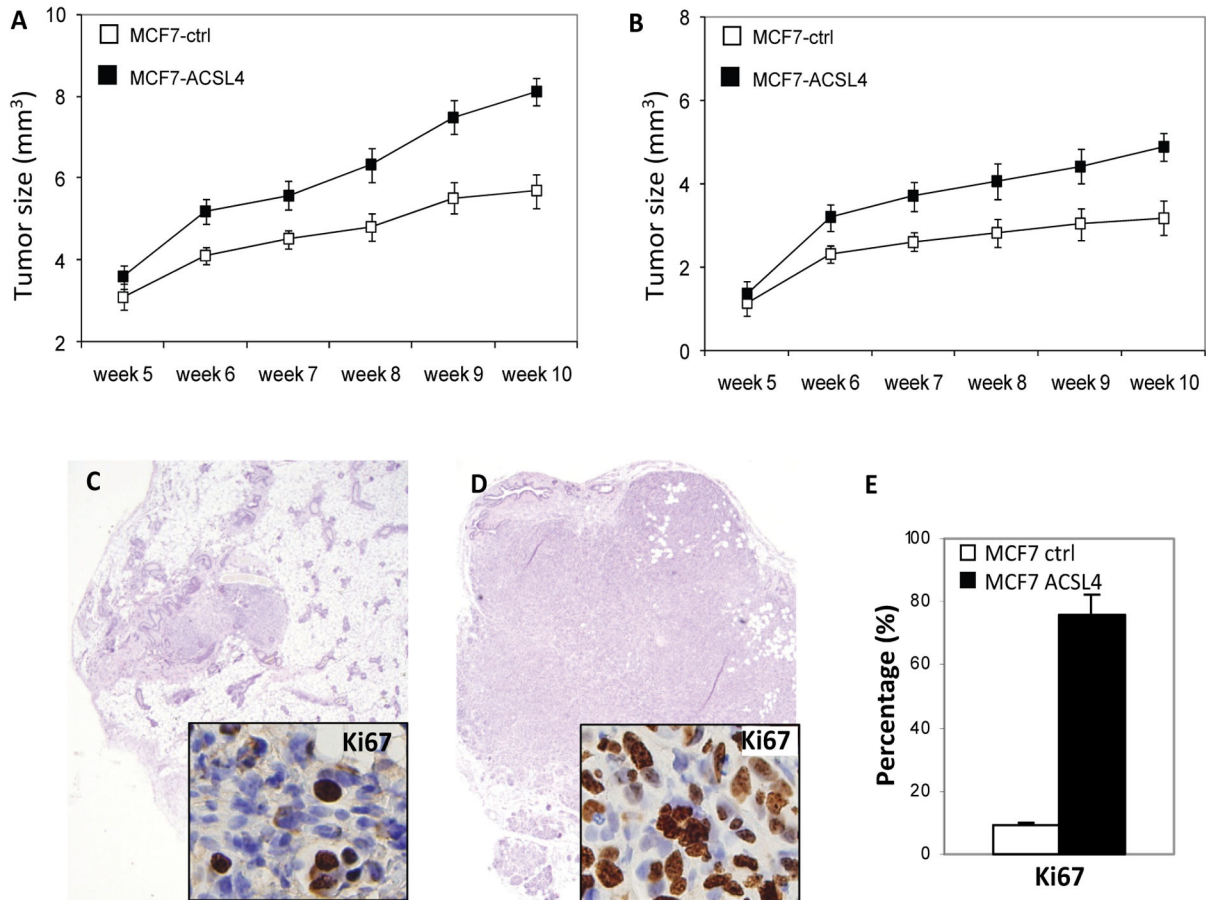


Figure 6. Comparison of the growth of control and ACSL4-MCF-7 cell tumors in nude mice. Nude mice, either intact (A) or ovariectomized (B) were injected with 1×10^7 MCF-7 vector control- (open circles) or ACSL4-transfected (open squares) cells. Each group contained 10 animals. The differences between the curves were significant (A, $p < 0.0001$ and B, $p < 0.0001$). Panel C and D depict H and E stained samples from control and ACSL4-transfected tumors, respectively. Staining for Ki67 is shown in the inset of C and D. Panel E compares the percentage of cells in each sample that stain positively for Ki67. Three sections of each slide were analyzed to determine the significance of the difference ($p = 0.001$).

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treatment with triacsin C [7]. ACSL5 has previously been reported to function as a cancer survival factor [32,48]

Not only is ACSL4 expression able to reduce ER expression, but an siRNA-mediated reduction of ER in MCF-7 cells induces expression of ACSL4 as shown in Figure 5. Results shown in Figure 5 were derived from microarray data reported by Al-Saleh et al [31]. Lastly, the proliferative effect of ACSL4 is also observed in a xenograft mouse model utilizing both intact and ovariectomized nude mice (Figure 6). Since nude mice have low levels of endogenous estrogen, and the intact mice were not treated with exogenous estrogen, it is not surprising that there is little difference observed between intact and ovariectomized mice. It will be interesting to see whether estrogen supplementation in the ovariectomized model inhibits growth of ACSL4-transfected MCF-7 cells, as is the case *in vitro*.

In the HER2-overexpressing cell line, SKBr3, stable expression of ACSL4 results in a slight increase in the rate of growth (Figure 7), as well as reduced sensitivity to treatment with lapatinib (Figure 8D), supporting the hypothesis that simultaneous expression and overexpression of both ACSL4 and HER2, respectively, are indicative of resistance to HER2-based therapy. Estrogen appeared to inhibit growth in the experiment shown in Figure 7. Further experimentation will be required to determine the significance of this inhibition in the absence of ER expression.

The effect of ACSL4 expression on overall gene expression was evaluated by microarray studies in two different cell lines: a conditional and stable MCF-7 model, as well as a stable SKBr3 model. Table S1 details the common affected genes across the three models, and Table S2 lists common affected pathways. Table 6 lists those shared affected genes across the three models that were decreased by $\geq 50\%$ or increased by

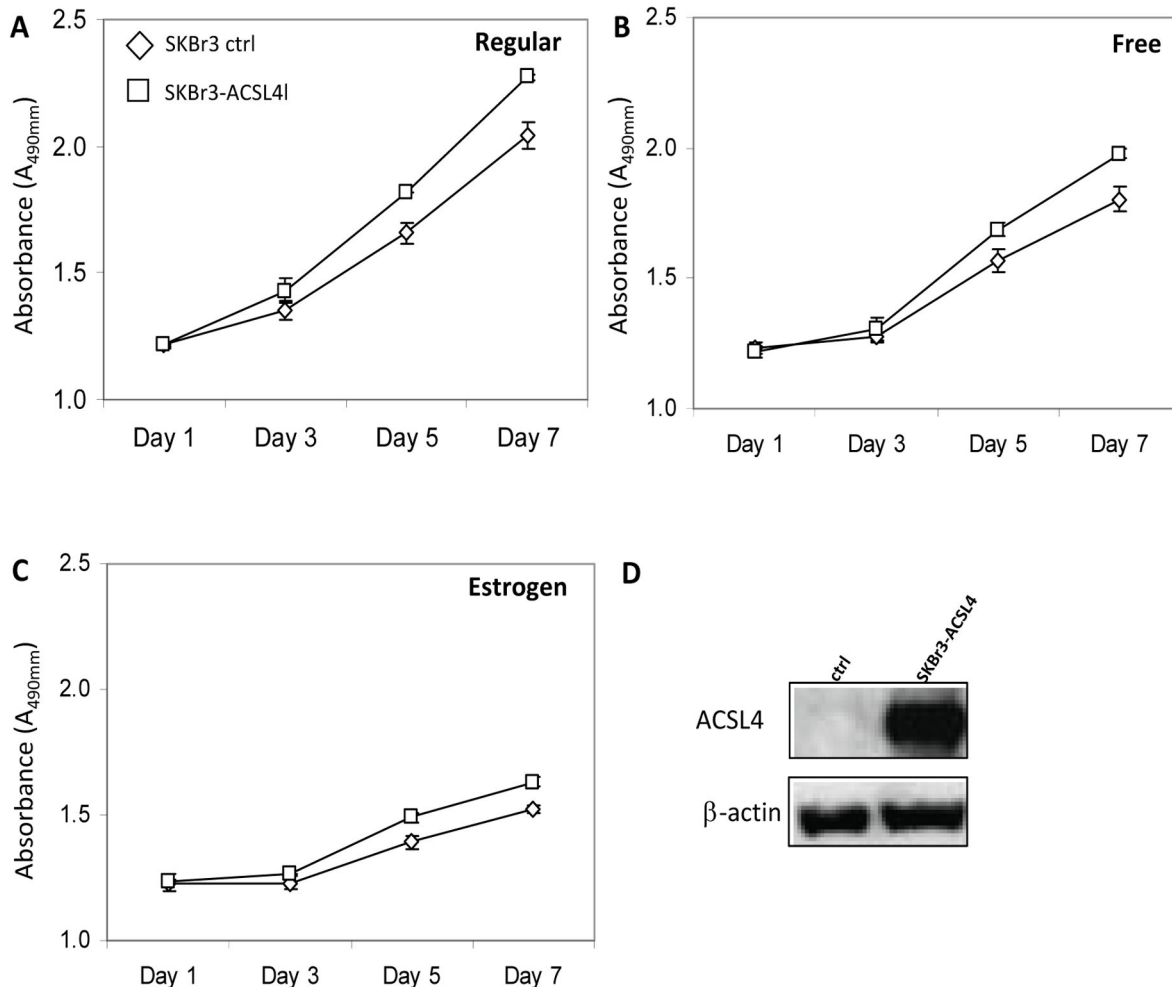


Figure 7. Effect of ACSL4 on growth of SKBr3 cells. Control- and ACSL4-transfected SKBr3 cells were cultured in complete medium (A), or phenol red-free, charcoal-stripped medium without (B) or with (C) added estrogen. Values shown are the means of triplicate determinations \pm 1SD. The differences between the curves are significant: **A**, $p < 0.0001$; **B**, $p < 0.0001$; **C**, $p < 0.0001$. Panel **D**, immunoblot analysis of whole cell lysates for expression of ACSL4 in control and ACSL4-transfected cells.

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$\geq 100\%$. The only gene whose expression was so affected was AUTS2, an effect validated by RT-PCR (Figure 9A). The function of this gene is thought to be associated with neural development, and mutations in AUTS2 have been linked to autism [35,49], although the exact nature of its activity remains unknown. The prevalence of autism in males suggests involvement of the X chromosome, and the association between X-linked mental retardation, a syndrome associated with mutations in ACSL4, which is located on the X-chromosome [34], and autism support the finding reported here that ACSL4 expression regulates AUTS2 expression.

The ability of ACSL4 to cause a decrease in X inactive specific transcript (XIST) in both MCF-7 models is of interest in light of the data implicating a possible role for this RNA moiety in the genesis of breast cancer [50]. Like ACSL4, XIST is located on the X chromosome.

When common affected pathways are analyzed, it becomes clear that expression of ACSL4 causes a general increase in cellular metabolism, including RNA and DNA synthesis, as well as a decrease the expression of pathways involved in signal transduction and cytoskeletal organization.

Table 9 lists changes in pathway-associated protein expression or phosphorylation state as a function of ACSL4 expression in MCF-7 and SKBr3 cells. Changes in p-PKC α , and β -catenin were validated (Figure 9C). The increase in β -catenin in SKBr3 cells was also verified by immunofluorescence, and it was noted that there was increased nuclear localization as a result of ACSL4 expression (Figure 9D).

Recent studies have addressed the mechanism by which ACSL4 induces an aggressive phenotype in breast cancer [12,47]. ACSL4 is an enzyme in the lipogenic pathway that supplies activated fatty acids for use in glycerolipid synthesis

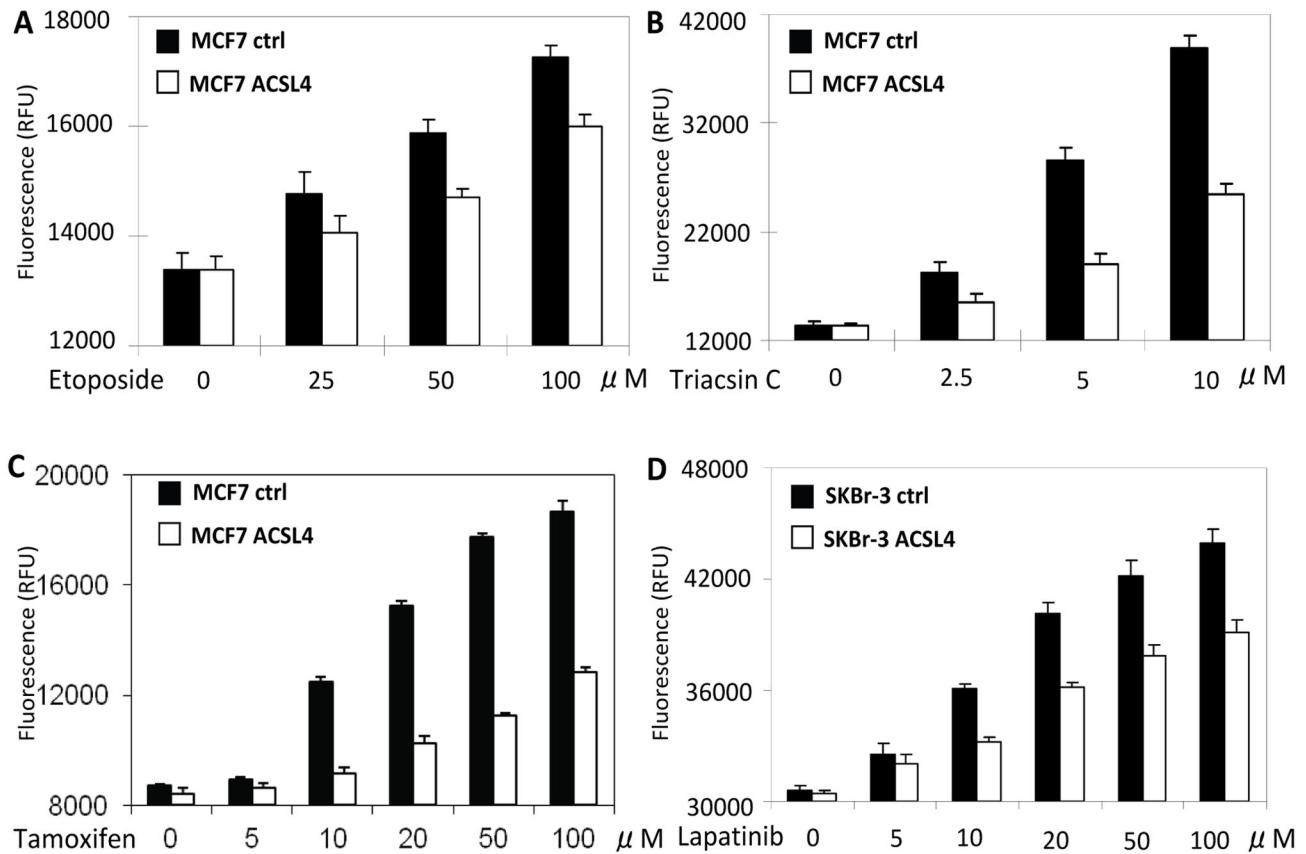


Figure 8. Effect of ACSL4 expression on apoptosis in MCF-7 and SKBr3 cells. Control and ACSL4-transfected MCF-7 cells were treated with varying doses of etoposide (A), triacsin C (B) or tamoxifen (C) for 48 hours and apoptosis measured as described in the text. For Panel D, control and ACSL4-transfected SKBr3 cells were treated with lapatinib for 48 hours and apoptosis measured as described in the text. Results shown are the means of triplicate determinations \pm 1SD. Significance of the differences are A, $p=0.027$; B, $p=0.012$; C, $p=0.005$, D, $p=0.008$.

doi: 10.1371/journal.pone.0077060.g008

Table 6. Shared affected genes: Comparison across all three experiments.

Probe Set ID	Gene Symbol	FC MCF-7-1 ¹	p-value	FC MCF-7-2 ²	p-value	FC SKBr3	p-value
212599_at	AUTS2	-2.48	7.16E-04	-2.50	7.71E-03	-2.41	3.04E-04

Microarray data generated as described in the text. Full results can be found in table S1.

1. Compares conditional, doxycycline-induced expression of ACSL4 in MCF-7 cells with doxycycline-treated control-transfected cells.

2. Compares control vector and ACSL4-transfected MCF-7 cells

FC = fold change

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and in β -oxidation. There is even some evidence that activated fatty acids (fatty acyl-CoAs) function as transcription factors [51]. The preference of this enzyme for arachidonic acid as a substrate has lead to the hypothesis that ACSL4 expression decreases the availability of free arachidonic acid for conversion to leukotrienes and eicosanoids, and thus functions to impede the production of these compounds, a suggestion that has been supported by data [13,52]. In the case of breast

cancer, however, ACSL4 has been demonstrated to increase production of lipoxygenase products, an effect attributed to increased uptake of AA-CoA into the mitochondria, followed by regeneration of free AA for subsequent conversion to prostaglandins [12]. ACSL4 expression also induces increased expression of COX-2. Thus ACSL4 activity increases the pool of free AA available for conversion to prostaglandins, as well as increasing enzyme activity (COX-2) involved in prostaglandin

Table 7. Shared affected genes: Comparison of conditional and stable induction of ACSL4 in MCF-7 cells.

Probe Set ID	Gene Symbol	FC MCF-7-1 ¹	p value	FC MCF-7-2 ²	p value
212599_at	AUTS2	-2.48	7.16E-04	-2.50	7.71E-03
207886_s_at	CALCR	-3.11	3.61E-03	-2.13	1.79E-03
224994_at	CAMK2D	-2.87	5.45E-04	-4.18	4.30E-03
209479_at	CCDC28A	2.03	1.29E-05	2.07	2.81E-02
231766_s_at	COL12A1	-5.22	1.04E-03	-3.44	6.23E-04
224822_at	DLC1	-2.18	5.02E-05	-2.31	6.47E-03
230263_s_at	DOCK5	-2.01	4.45E-03	-2.11	2.18E-04
1555606_a_at	GDPD1	4.77	7.83E-04	2.68	3.86E-02
214469_at	HIST1H2AE	6.03	6.24E-03	2.05	4.72E-02
205842_s_at	JAK2	-2.69	1.60E-02	-2.04	3.89E-02
201505_at	LAMB1	-3.99	1.01E-02	-2.29	1.23E-02
227761_at	MYO5A	-2.70	1.16E-03	-2.32	1.07E-02
213988_s_at	SAT1	2.18	4.30E-05	4.06	1.28E-02
226051_at	SELM	2.03	9.82E-03	2.63	4.78E-03
210664_s_at	TFPI	2.20	2.32E-03	2.80	3.05E-02
203887_s_at	THBD	-2.38	3.17E-04	-2.16	2.27E-02
227671_at	XIST	-12.40	3.05E-07	-77.05	1.36E-02

Microarray data generated as described in the text. Full results can be found in table S1.

1. Compares conditional, doxycycline-induced expression of ACSL4 in MCF-7 cells with doxycycline-treated control-transfected cells.

2. Compares control vector and ACSL4-transfected MCF-7 cells

FC = fold change

doi: 10.1371/journal.pone.0077060.t007

Table 8. Shared affected genes: Comparison of stable induction of ACSL4 in MCF-7 and SKBr3 cells.

Probe Set ID	Gene Symbol	FC MCF-7-2	p value	FC SKBr3	p value
212599_at	AUTS2	-2.50	7.71E-03	-2.41	3.04E-04
235626_at	CAMK1D	-2.84	6.88E-05	-2.00	2.80E-05
236313_at	CDKN2B	-2.74	4.31E-04	-2.26	7.46E-05
229088_at	ENPP1	2.08	4.82E-04	2.53	1.57E-04
218796_at	FERMT1	-2.01	1.92E-03	-3.09	2.01E-03
203710_at	ITPR1	-3.17	1.20E-02	-2.68	1.51E-05
203939_at	NT5E	-3.81	2.20E-04	-4.86	3.70E-04
208510_s_at	PPARG	2.34	1.96E-02	2.17	1.56E-02
228396_at	PRKG1	-2.21	9.74E-03	-4.04	1.60E-02
223168_at	RHOU	-2.35	3.64E-02	-3.18	9.16E-07

Microarray data generated as described in the text. Full results can be found in table S1.

FC = fold change

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synthesis. The result is a more aggressive breast cancer phenotype. In prostate cancer, induction of COX-2 has been shown to be effected by addition of AA via the PI3K/AKT pathway [53]. Whether or not this effect involves ACSL4 activity remains to be determined.

The unique subcellular localization of ACSL4 to peroxisomes and mitochondria-associated endoplasmic reticulum membranes suggests a possible role for this enzyme in facilitating oxidation of free fatty acids as a source of energy in proliferating tumor cells. Thus both substrate specificity and subcellular localization are currently under investigation in our

laboratory in order to determine their role in mediating the effects of ACSL4 expression on breast cancer phenotype.

Breast cancer is not the only cancer demonstrated to differentially express ACSL4. It has previously been reported that ACSL4 expression is associated with the malignant phenotype in both liver and colon cancer and functions to modulate proliferation [10,11]. In breast cancer, this association is limited to the most aggressive forms of the disease, with the more benign, receptor positive cancers being negative for ACSL4 expression. Since normal mammary epithelium is predominantly receptor-negative, ACSL4 expression is positive for normal compared with most (i.e. receptor-positive) breast

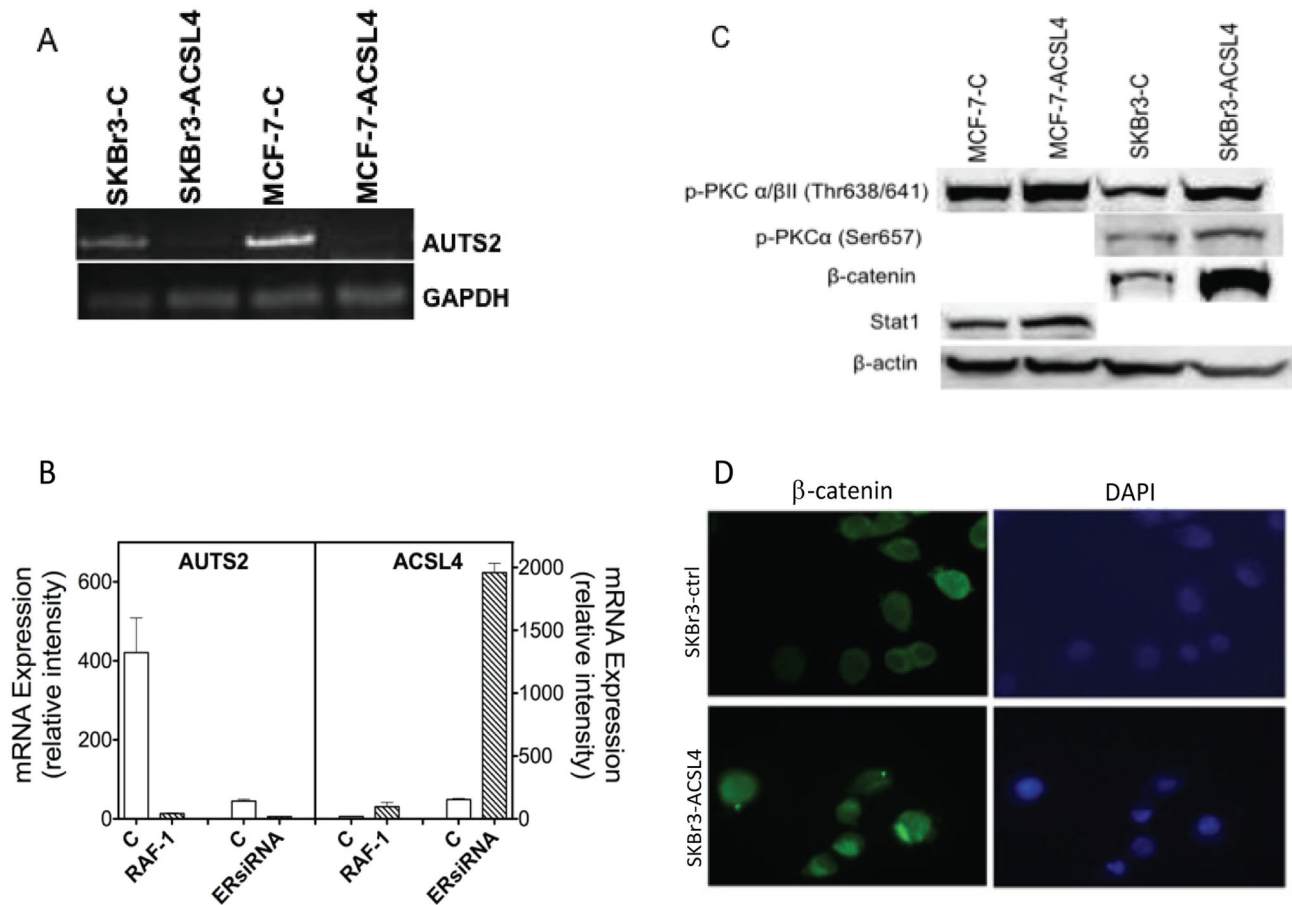


Figure 9. Validation of microarray and proteomic pathway analysis data. Panel **A** illustrates results from a semi-quantitative RT-PCR analysis of control- and ACSL4-transfected MCF-7 and SKBr3 cells. Both transfections were carried out with lentivirus vectors as described in the text. Panel **B** documents changes in AUTS2 expression as a result of transfection of MCF-7 cells with either RAF-1 or siRNA directed against ER. Values shown represent the means of three determinations \pm 1SD. The differences between controls and transfected cells are significant, $p=1.0E-03$ and $p=9.0E-06$, respectively. Data were taken from microarray studies GDS1925 and GDS4061 deposited at <http://www.ncbi.nlm.nih.gov/geo/>. Panel **C** depicts an immunoblot analysis of selected pathway proteins. Fold changes observed for expression of p-PKCα/βII (Thr638/641) are 1.4 in MCF-7 cells and 1.9 in SKBr3 cells; for expression of p-PKCα is 2.0 in SKBr3 cells; for expression of β-catenin is 35 in SKBr3 cells; and for expression of stat1 is 1.1 in MCF-7 cells. Panel **D** shows immunofluorescent staining of β-catenin in control and ACSL4-transfected SKBr3 cells. Methods were as described in the text.

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cancer tissue. The precise role of ACSL4 activity in generating the malignant phenotype seen in colon, liver and aggressive breast cancers remains to be determined. Its utility as a biomarker for differentiation of breast cancer molecular subtypes and for prediction of treatment response and prognosis is supported by the evidence presented here and in prior studies by our group and others. Further work will be required to demonstrate that routine measurement of ACSL4 in breast tumor samples might obviate the need for hormone/HER2 receptor measurements in cases where ACSL4 is positive, or be useful as a marker, in addition to receptor status, in making treatment decisions. Of particular interest is a possible role for ACSL4 as a mediator of the racial disparity

observed in the increased prevalence of TNBC in the African American population. Microarray analyses performed on liver tissue from patients undergoing weight loss surgery revealed elevated levels of ACSL4 mRNA in African Americans when compared to Caucasians [54].

Conclusions

An analysis of the expression of ACSL4 in breast cancer indicates that there is an inverse relationship between the presence of this lipid metabolic enzyme and the steroid hormone/HER2 receptor status of the sample. In a study of 71 different breast cancer cell lines, ACSL4 status predicted

Table 9. PPAA analysis of pathway protein expression as a function of ACSL4 expression.

	MCF-7 Cells		SKBr3	
	Fold Change*		Fold Change	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
Protein				
p-PKC α (Ser657)	NC	NC	1.59	1.83
p-PKC α/β II (Thr638/641)	1.66	2.04	2.16	1.78
Stat1	1.22	2.14	NC	NC
cdc25B	0.48	0.83	NC	NC
EGFR	1.09	2.32	NC	NC
Hsp90	NC	NC	0.35	0.74
PCNA	NC	NC	0.38	0.78
p38 β	NC	NC	0.30	0.67
β -catenin	NE	NE	20.93	16.83
XIAP	0.51	0.52	NC	NC
OPN	NE	NE	1.32	7.53
WT1	NC	NC	0.47	0.76
NFkBp50	NC	NC	0.57	0.49
Calretinin	0.82	0.14	NC	NC
ICAM-1	0.37	0.26	NC	NC
c-Flip	NC	NC	0.36	0.53
Rab 7	1.54	1.82	2.53	2.18
Bak	1.92	2.03	NC	NC
Nkx-3.1	NC	NC	1.72	1.76
RIP	0.48	0.66	NC	NC
ERCC1	NC	NC	2.77	1.38
L-Selectin	2.28	3.01	2.48	1.45
Cytokeratin 18	NC	NC	0.55	0.47
FAH	NC	NC	3.31	1.49
LSD1	NC	NC	2.23	1.62
LKB1	NC	NC	0.77	0.43
PEDF	NC	NC	0.41	0.10
SPAK	NC	NC	0.45	0.04
ADH	0.52	0.23	0.69	0.43

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QNBC status with a sensitivity of 78% and a specificity of 86%. Higher ACSL4 expression was also associated with TNBC tumor samples. In cases where ACSL4 expression status failed to predict receptor status, it is possible that a sub classification based on ACSL4 status might have prognostic or therapeutic implications. Including ACSL4 expression data in gene signatures designed to assess intrinsic molecular subtype might improve the accuracy of these determinations. There is also the possibility that adding ACSL4 measurements to current measurements of sex steroid and HER2 receptors would increase the predictive value of current protocols. Studies assessing the effect of ACSL4 expression in breast cancer cell lines suggest that ACSL4 functions as a mediator of hormone independence and resistance to hormonal and chemotherapy. Thus simultaneous expression of ACSL4 and a receptor might serve as an indicator of resistance to targeted therapies, while it is possible that receptor-negative tumors that are also ACSL4-negative might be less aggressive than ACSL4-positive tumors and be amenable to receptor-based

therapies. In essence, ACSL4 status may be a biomarker that further defines receptor status. Given that receptor status does not always predict intrinsic molecular subtype, inclusion of ACSL4 status as a biomarker might prove useful as a prognostic and therapeutic guide. In addition, ACSL4 itself might serve as a useful target for development of future therapies.

Supporting Information

Table S1. Common affected genes. Results from gene expression studies comparing control-transfected and ACSL4-transfected MCF-7 and SKBr3 cells. Methods were as described in the text. MCF-7 cells were transfected with either a doxycycline-inducible expression vector (MCF-7-1) or a lentiviral expression vector (MCF-7-2). SKBr3 cells were transfected with a lentiviral vector.
(XLS)

Table S2. Common affected pathways. An analysis of the results detailed in table S1 using methods described in the text. (XLS)

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Conceived and designed the experiments: MEM PL X. Wu. Performed the experiments: X. Wu YL FY LHW MTM GD JZ MEM XD. Analyzed the data: MEM JW X. Wu X. Wen MTM DYZ SA BS JZ PL FY LHW GD XD. Contributed reagents/materials/analysis tools: MEM PL JZ DYZ JW X. Wen. Wrote the manuscript: MEM PL X. Wu SA BS JZ MTM DYZ GD.

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